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(54) Title: METHOD FOR POTENTIATING AND INHIBITING INSULIN-LIKE GROWTH FACTOR ACTIVITY

(57) Abstract

Insulin-like growth factor I and II binding protein have potentiating and inhibiting activities. Cloning vectors and portable DNA sequences are disclosed.

METHOD FOR POTENTIATING AND INHIBITING INSULIN-LIKE
GROWTH FACTOR ACTIVITY

BACKGROUND OF THE INVENTION

This invention relates to methods for potentiating
the activity of insulin-like growth factor. This invention
also relates to a human insulin-like growth factors I and II
(ICG-I and II) binding protein (stimulatory form) which has
this activity and to methods for producing this binding pro-
tein. The binding protein (stimulatory form) has utility in
potentiating follicular development in vivo and in vitro, po-
tentiating wound and burn healing, ulcer healing, and for
re-epithelialization of damaged tissue of the kidney, lung or
skin. The portein may also have utility in potentiating
healing or damaged neurons and oligodendrocytes.

In addition, this invention relates to a human
insulin-like growth factor binding protein (inhibitory form).
The IGF binding protein (inhibitory form) may be used to in-
hibit tumor growth, inhibit the progression or growth of
atherosclerotic plaques, and inhibit diabetic retinopathy,
and inhibit pulmonary fibrosis.

Human insulin-like growth factor I (IGF-I), also
termed somatomedin-C, is a growth hormone dependent growth
factor that circulates in blood and is synthesized in many
tissues. Growth factor IGF-I is useful as an additive to
cell culture media to facilitate in vitro cell growth. Like
other growth factors that circulate in plasma, such as EGF,
the insulin-like growth factors are bound to a binding pro-
tein that forms a carrier protein-IGF-I complex and is
believed to serve a transport function. Partially purified
forms of this binding protein previously have been shown to
inhibit the insulin-like actions of IGF-I. An acid stable
binding subunit of this complex has been purified from blood,
and its secretion can be stimulated by growth hormone.

Human insulin-like growth factor II is weakly or
not at all growth hormone dependent and is synthesized in
many tissues. Like IGF-I, it circulates bound to a binding
protein that is believed to serve a transport function.

In contract to the above growth factor binding pro-
teins, extracellular fluids such as spinal, lymph, and

amniotic fluids, and tissue extracts of brain, placenta, and
pituitary contain forms of IFG binding protein that have dif-
ferent molecular weights and are not growth hormone depen-
dent. They bind both IGF-I and IFG-II with affinities in the
10^{10} to 10^9 m^{-1} range. An IGF binding protein has also been
shown to be secreted by some cell types in culture including
human fibroblasts and MDA-231 cells. Impure preparations of
the protein from amniotic fluid and a similar protein that is
secreted by rat liver cells, have been shown to inhibit the
effects of IGF-I and IGF-II on fibroblast DNA synthesis and
on sulfate incorporation into cartilage.

However, all of the prior human proteins were actu-
ally mixtures of more than one protein. For the first time,
the present inventors have developed a method to separate
these binding proteins. It has been found that the protein
mixture from amniotic fluid actually contained at least two
protein constituents. These protein constituents were found
to have greatly different activity, one being capable of
stimulating or enhancing the activity of IGF-I and IGF-II and
one capable of inhibiting the growth factor activity of IGF-I
and IGF-II. These two constituent proteins, whose isolation
to substantial purity is described herein for the first time,
are referred to for the purposes of this application as IGF
binding protein (stimulatory form) and IGF binding protein
(inhibitory form), respectively. Experiments have indicated
that the proteins may differ in the type of post-
translational modifications, possibly disulfide bond arrange-
ments. Such changes may lead to differences in degree of
aggregation that may account for the observed differences in
cell or matrix binding of the IGFBPs and their biological
activity.

In addition to the above amniotic fluid IGF binding
proteins, an IGF binding protein has been isolated by the
present inventors from human fibroblast conditioned media.
This form as IGB binding protein potentiates the action of
IGF like the stimulatory form from amniotic fluid. It cross-
reacts with polyclonal antibodies to the IGF (stimulatory
form) but may not be the same protein.

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The purified IGF binding proteins (stimulatory form) have an estimated molecular weight between 32-38K daltons. These purified proteins, when adherent to cell surfaces, cause an increase in the amount of IGF that binds to the IGF receptor and to cell surfaces. Surprisingly, this increased amount of bound IGF results in a synergistic potentiation of the IGF activity.

The substantially purified IGF binding protein (inhibitory form) also has a molecular weight of 32-38 KD but does not adhere to cell surfaces and elutes later on DEAE-cellulose columns.

In the discussion which follows, reference to IGF-I binding protein or to IGF-II binding protein should be considered to refer to either or both species, unless the context indicates otherwise. Also, the abbreviation IGFBP may be used to refer to an IGF binding protein.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide compounds capable of increasing the activity of certain growth fatos. In partiuclar, it is an object of the present invention to provide compounds capable on increasing the activity of insulin-like growth factor type I and II (IGF-I and IGF-II).

It is also an object of the present invention to provide compounds capable of preventing the action of IGF-I and IGF-II.

It is an additional object of the present invention to provide methods for obtaining each of these proteins in substantially purified form. The methods which are provided for the purification of these compounds allow the compounds to retain their relative IGF-affecting activity. The methods are to include both methods for purifying the naturally-ocurring proteins and recombinant-DNA methods for producing IGFBP in other cells, or in cultured cells, and for purifying these proteins.

To accomplish these objects and in accordance with the purposes of the present invention, a method for

potentiating the cell growth effects of IGF is provided. This method comprises:

      (a)   Obtaining IGF binding protein (stimulatory form) in a substantially purified form; and

      (b) Exposing the cells whose growth is to be stimulated to the IGF binding protein (stimulatory form) and to IGF.

      To also accomplish the objects and also in accordance with the purposes of the present invention, a method of reducing the cell growth effects of IGF is provided. This method comprises:

      (a)   Obtaining IGF binding protein (inhibitory form) in a substantially purified form; and

      (b) Exposing the cells whose growth is to be inhibited to the IGF binding protein (inhibitory form).

      To further accomplish the objects of the present invention and in further accord with the purposes hereof, methods for purifying the IGF binding proteins (stimulatory form) and (inhibitory form) are provided. These methods provide both forms of IGF binding protein in a substantially purified state which retains its IGF affecting activity.

      It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one embodiment of the invention and, together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts potentiation of DNA synthesis in porcine aortic smooth muscle cells (A) chick (B) mouse (C) embryo fibroblasts by IGF-I and binding protein. Smooth muscle cells were subcultured on microtest 96-well plates plating at 8,000 cells/well and allowing 5 days for the cells to become quiescent. Prior to the analysis, the cultures were washed two times with serum free DMEN followed by addition of the

the invention and, together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts potentiation of DNA synthesis in porcine aortic smooth muscle cells (A) chick (B) mouse (C) embryo fibroblasts by IGF-I and binding protein. Smooth muscle cells were subcultured on microtest 96-well plates plating at 8,000 cells/well and allowing 5 days for the cells to become quiescent. Prior to the analysis, the cultures were washed two times with serum free DMEN followed by addition of the test substances in 0.2 ml DMEM supplemented with 0.5 uCi 3H-thymidine 1% human platelet poor plasma (PPP). Chick embryo fibroblasts (2nd passage) were subcultured on 96-well microtest plates (Falcon 3004) 3,000 cells/well in DMEM supplemented with 10% FBS. Five days after plating the quiescent monolayers were exposed to the test factors listed plus 0.5 uCi ^{3}H-thymidine and 1% PPP. Mouse embryo fibroblast cultures between the 3rd and 5th passages were used and the experiments were performed as described for chick embryo fibroblasts except that the cells were plated at 8,000 cells/well. All cultures that were exposed to increasing concentrations of human serum, insulin, IGF-I, IGF-I plus IGF-I binding protein (stimulatory form) (100 ng/ml), or insulin and IGF-I binding protein (stimulatory form), for 36 hours and ^{3}H-thymidine incorporation were quantitated. The results are expressed as the mean of triplicate cultures.

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^{3}H-thymidine incorporation was determined asd described in Fig. 1. The results are expressed as the mean ^{3}H-thymidine incorporation of triplicate cultures.

Figure 4 depicts growth of cultured fibroblasts and smooth muscle cells in response to IGF-I and IGF-I binding protein (stimulatory form). Porcine smooth muscle cells (A) and human fibroblasts (B) were plated at 15,000 cells/well on 24-well plates (Falcon, 3004), in MEM plus 10% calf serum (fibroblasts) or DMEM plus 10% fetal calf serum (smooth muscle cells). After two hours, the media were changed to MEM or DMEM containing 1% PPP. Following an additional 12 hour incubation, the media were removed and test reagents were added to 0.5 ml DMEM or MEM containing 1% PPP. Following a 48 hour incubation, cell number was determined. The results are expressed as the + 1SD of quadruplicate cultures.

Figure 5 shows the construction of an expression vector for IGFBP in higher eukaryotic cells.

Figure 6 is the amino acid and nucleotide sequence of IGFBP with a 24 amino acide signal sequence.

Figure 7 depicts the creation of plasmid pJU1020 containing the IGFBP coding sequence.

Figure 8 depicts the creation of plasmid pJU1021 containing the IGFBP coding sequence.

Figure 9 depicts the creation of plasmid pJU1022 containing the IGFBP coding sequence.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of this invention, which, together with the drawings and the following examples, serve to explain the principles of the invention.

As noted above, the present invention relates in part to a method for potentiating the effect on cells of insulin-like growth factors (IGF). It is known that IGF functions as growth factors by increasing the rate of DNA synthesis. The present inventors have obtained, in a substantially purified form, a protein which potentiates the growth promoting effects of IGF-I. This protein has been

named IGF binding protein (stimulatory form). The poten-
tiating effect of IGF binding protein (stimulatory form) on
the ability of IGF-I to promote cell growth is depicted in
Figures 1 through 4.

To obtain this potentiating effect, the IGF binding
protein (stimulatory form) is obtained in a substantially
purified form. In one embodiment, this substantially
purified IGF binding protein (stimulatory form) is first
adhered to the surfaces of the target cells. Increased. The
target cells with the adhered IGF binding protein
(stimulatory form) are then exposed to IGF-I. The exposure
to IGF-I, coupled with the present of the adhered IGF binding
protein, results in at least about a four-fold increase in
cellular growth rate whereas exposure to IGF-I alone resulted
in about a 25% increase in cellular growth rates, using
smooth muscle cells.

In a second embodiment, the IGF binding protein
(stimulatory form) and the IGF-I are pre-mixed prior to their
addition to the target cells.

The IGF binding protein (stimulatory form) of the
present invention is obtained, in one embodiment, in a sub-
stantially purified form by isolation from human tissues. In
particular, a substantially purified IGF binding protein
(stimulatory form) may be isolated from human extracellular
fluids. These extracellular fluids include amniotic and spi-
nal fluids. In a preferred form of this embodiment, the IGF
binding protein (stimulatory form) is isolated from human
amnionic fluid. A detailed methodology for the isolation of
this particular IGF binding protein (stimulatory form) from
extracellular fluids is set forth in Example 1, which fol-
lows.

In an alternate embodiement, the IGF binding pro-
tein (stimulatory form) of the present invention may be iso-
lated in a substantially purified form from media conditioned
by human cell lines. The cell lines include, but are not
limited to, fibroblast cell lines, such as GMIO, and breast
carcinoma cell lines such as MDA-231. A detailed methodology

for the isolation of this particular IGF binding protein
(stimulatory form) from fibroblast conditioned media is set
forth in Example 5, which follows. This second IGF binding
protein (stimulatory form) is closely related to but not
identical to the protein isolatable from extracellular
fluids.

When either type of IGF binding protein
(stimulatory form) is being isolated, a series of tests have
been developed to assist in determining that the correct pro-
tein has been isolated. These tests may be used indepen-
dently or may be used in some combination to confirm the iso-
lation of IGF binding protein (stimulatory form).

The first of these tests is to determine whether
the isolated protein can bind IGF-I. Ine method for making
this determination is described in Clemmons, D. in J. Clin.
Invest. 77:1548-1556 (1986), the disclosure of which is
expressly incorporated herein by reference. A second test is
the ability to promote the effects IGF-I in a synergistic
manner. One method for conducting this test is set forth in
Example 2.

A third test is the ability of the isolated protein
to react with an antibody raised against IGF-I binding pro-
tein (stimulatory form). In particular, polyclonal
antibodies may be raised against this protein by any of the
methods known to those of ordinary skill in the art, particu-
larly in light of the teachings contained herein and used to
confirm the identity of binding protein.

In the above description, the term "substantially
purified" has been used. For the purposes of this applica-
tion, this term is intended to mean that the protein is at
least about 50% free of contaminants as determined by SDS
PAGE. Preferably, the protein is at least about 75% free of
contaminants and most preferably at least about 90% free of
contaminants. It should be noted that the IGF binding pro-
tein previously isolated occurs naturally as a mixture of IGF
binding protein (inhibitory form) and (stimulatory form).
Thus, while the IGF binding proteins described herein are

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"substantially pure" when at least about 50% free of
contaminants, these binding proteins should be at least about
75% separated from each other. The isolation and purifica-
tion of the IGF binding proteins of the present invention are
described in Example 1.

The sequence of an IGF binding protein is described
in Example 3. Isolation of the gene encoding an IGF binding
protein is described in Example 4.

In another embodiment, the present invention re-
lates to the portable DNA sequences encoding insulin-like
growth factor binding proteins. These portable DNA sequences
are capable of directing production of human IGF binding pro-
teins in a variety of host microorganisms and eukaryotic
cells. "Portable DNA sequence" in this context is intended
to refer either to a full length DNA clone or synthetically-
produced analog or any combination of the two. For the pur-
poses of this specification, "human-like growth factor bind-
ing proteins" are intended to mean the primary structure of
the proteins as defined by the codons present in the
deoxyribonucleic acid sequence which directs intracellular
production of the amino acid sequences, and which may or may
not include post-translational modifications. The "human
insulin-like growth factor binding proteins produced by the
present invention are at least 50%, preferably at least 60%
and most preferably at least 80% identical to the naturally-
occurring human proteins. The percent identity as discussed
herein is calculated as the percentage of amino acid residues
found in the smaller of the two sequences that align with
identical amino acid residues in the sequence being compared
when four gaps per 100 amino acids are allowed to assist in
alignment.

In a preferred embodiment, the portable DNA se-
quences are capable of directing production of an IGF binding
protein. In a particularly preferred embodiment, the porta-
ble DNA sequences are capable of directing production of an
IGF binding protein biologically equivalent to that previous-
ly isolated from human amniotic fluid or fibroblast

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conditioned medium. By "biologically equivalent," as used herein the specification and claims, it is meant that the IGF binding protein produced using a portable DNA sequence of the present invention is activatable to a form capable of inducing a potentiated or inhibited cell growth activity qualitatively and quantitatively equivalent to that produced by IGF binding proteins isolated from human amniotic fluid or fibroblast conditioned media.

As noted previously, the portable DNA sequences of the present invention may be synthetically created. It is believed that the means for synthetic creation of these poly-nucletide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings con-tained herein. As an example of the current state of the art relating to polynucleotide synthesis, one is directed to Matteucci, M.D. and Caruthers, M.H., in J. Am. Chem. Soc. 103:3185 (1981) and Beauchee, S.L. and Caruthers, M.H. in Tetrahedron Lett. 22:1859 (1981), each of which is specifi-cally incorporated herein by reference.

Additionally, the portable DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a poly-nucleotide which occurred in nature and which has been iso-lated and purified for the first time by the present inven-tors. In one embodiment, the portable DNA sequence is a restriction fragment isolated from a CDNA library. In this preferred embodiment, the cDNA library is created as described in Example 4.

In an alternative embodiment, the portable DNA se-quence is isolated from a human genomic library. An example of such a library useful in this embodiment is set forth by Lawn et al. in cell 15:1157-1174 (1978), specifically incor-porated herein by reference.

As noted above, the present invention relates to a series of vectors, each containing at least one of the porta-ble DNA sequences described herein. It is contemplated that additional copies of the portable DNA sequence may be includ-ed in a single vector to increase the host organism or

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eukaryotic cell's ability to produce large quantities of the desired IGF binding protein. In addition, the cloning vectors within the scope of the present invention may contain supplemental nucleotide sequences preceding or subsequent to the portable DNA sequence. The supplemental sequences are those that will not interefere with transcription of the portable DNA sequence and will, in some instances as set forth more fully below, enhance transcription, translation, or the ability of the primary amino acid structure of the resultant IGF binding protein to assume a functional tertiary form.

It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefor suitable for use in the present invention. These vectors are also contemplated as being within the scope of the disclosed series of cloning vectors into which the cDNA sequences may be introduced, along with any necessary operational elements, and. which altered vector is then included within the scope of the present invention and would be capable of being used in the recombinant-DNA method set forth more fully below. Certain preferred vectors with these characteristics are described in the Examples which follow.

I. ·   Expression of Genes for IGF Binding
          Proteins in Microorganism Cells

Sections A, B and C which follow immediately below refer to the production of portable DNA sequences in microorganism hosts.

          A.   Characteristics of Preferred
                Vectors for Microorganisms

Certain embodiments of the present invention are envisioned which employ known or currently undiscovered vectors which would contain one or more of the cDNA sequences described herein. ·In particular, it is preferred that these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stably maintained and propagated in the desired host; (3) be capable of being present in a high copy number in the

desired host; (4) possess a regulatable promoter positioned
so as to promote transcription of the gene of interest; (5)
have at least one DNA sequence coding for a selectable trait
present on a portion of the plasmid separate from that where
the portable DNA sequence will be inserted; and (6) a DNA se-
quence capable of terminating transcription.

In various preferred embodiments, these cloning
vectors containing and capable of expressing the cDNA se-
quences of the present invention contain various operational
elements. These "operational elements," as discussed herein,
include at least one promoter, at least one Shine-Dalgarno
sequence and initiator codon, and at least one terminator
codon. Preferable, these "operational elements" also include
at least one operator, at least one leader sequence for pro-
teins to be exported from intracellular space, at least one
gene for a regulator protein, and any other DNA sequences
necessary or preferred for appropriate transcription and
subsequence translation of the vector DNA.

Certain of these operational elements may be pres-
ent in each of the preferred vectors of the present inven-
tion. It is contemplated that any additional operational
elements which may be required may be added to these vectors
using methods known to those of ordinary skill in the art,
particularly in light of the teachings herein.

In practice, it is possible to construct each of
these vectors in a way that allows them to be easily iso-
lated, assembled and interchanged. This facilitates assembly
of numerous functional genes from combinations of these ele-
ments and the coding region of the cDNA sequences. Further,
may of these elements will be applicable in more than one
host. It is additionally contemplated that the vectors, in
certain preferred embodiments, will contain DNA sequences
capable of functioning as regulators ("operators"), and other
DNA sequenes capable of coding for regulator proteins.

1.   Regulators

These regulators, in one embodiment, will serve to
prevent expression of the cDNA sequences in the presence of

certain environmental conditions and, in the presence of
other environmental conditions, will allow transcription and
subsequent expression of the protein coded for by the cDNA
sequence. In particular, it is preferred that regulatory
segments be inserted into the vector such that expression of
the cDNA sequence will not occur, or will occur to a greatly
reduced extent, in the absence of, for example,
isopropylthio-beta-D-galactoside. In this situation, the
transformed microorganisms containing the cDNA sequence may
be growth at a desired density prior to initiation of the
expression of IGF binding protein. In this emodiment expres-
sion of the desired protein is induced by addition of a sub-
stance to the microbial environment capable of causing
expression of the DNA sequence after the desired density has
been achieved.

2.   Promoters

The expression vectors must contain promoters which
can be used the most organism for expression of its own pro-
teins. While the lactose promoter system is used commonly,
other microbial promoters have been isolated and character-
ized, enabling one skilled in the art to use them for expres-
sion of the recombinant IGF binding protein.

3.   Transcription Terminator

The transcription terminators contemplated herein
serve to stabilize the vector. In particular, those se-
quences as described by Rosenberg, M. and Court, D., in Ann.
Rev. Genet. 13:319-353 (1979), specifically incorported here-
in by reference, are contemplated for use in the present
invention.

4.   Non-Translated Sequence

It is noted that, in the preferred embodiment, it
may also be desirable to reconstruct the 3' or 5' end of the
coding region to allow incorproation of 3' or 5'
non-translated sequences into the gene transcript. Included
among these non-translated sequences are those which stabi-
lize the mRNA as they are identified by Schmeissner, U.,
McKenney, K., Rosenberg, M and Court, D. in J. Mol. Biol.

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176:39-53 (1984), specifically incorporated herein by refer-
ence.

5.   Ribosome Binding Sites

The microbial expression of foreign proteins re-
quires certain operational elements which include, but are
not limited to ribosome binding sites. That particular ele-
ment is a sequence which a ribosome recognizes and binds to
in the initiation of protein synthesis as set forth in Gold,
et al., Ann. Rev. Microbio. 35:557-580; or Marquis, D.M., et.
al, Gene 42:175-183 (1986), both of which are specifically
incorporated herein by reference.

The operational elements as discussed herein can be
routinely selected by those of ordinary skill in the art in
light of prior literature and the teachings contained herein.
General examples of these operational elements are set forth
in B. Lewin, Genes, Wiley & Sons, New York (1983), which is
specifically incorporated herein by reference. Various exam-
ples of suitable operational elements may be found on the
vectors discussed above and may be elucidated through review
of the publications discussing the basic characteristics of
the aforementioned vectors.

6.   Leader Sequence and
Translational Coupler

Additionally, it is preferred that DNA coding for
an appropriate secretory leader (signal) sequence be present
at the 5' end of the portable DNA sequence. The DNA for the
leader sequence must be in a position which allows the pro-
duction of a fusion protein in which the leader sequence is
immediately adjacent to and covalently joined to the inhib-
itor, i.e., there must be no transcription or translation
termination signals between the two DNA coding sequences.
The presence of the leader sequence is desired in part for
one or more of the following reasons. First, the presence of
the leader sequence may facilitate hose processing of the IGF
binding protein. In particular, the leader sequence may
direct cleavage of the initial translation product by a lead-
er peptidase to remove the leader sequence and leave a

polypeptide with the amino acide sequence which has potential
protein activity. Second, the presence of the leader se-
quence may facilitate purification of the IGF binding pro-
tein, through directing the protein out of the cell
cytoplasm. In some species of hose microorganisms, the pres-
ence of the appropriate leader sequence will allow transport
of the completed protein into the periplasmic space, as in
the case of some E. coli. In the case of certain E. coli,
Saccharomyces and strains of Bacillus and Pseudomonas, the
appropriate leader sequence will allow transport of the pro-
tein through the cell membrane and into the extracellular me-
dium. In this situation, the protein may be purified from
extracellular protein. Thirdly, in the case of some of the
proteins prepared by the present invention, the presence of
the leader sequence may be necessary to locate the completed
protein in an environment where it may fold to assume its
active structure, which structure possesses the appropriate
protein activity.

7.   Translation Terminator

The translation terminators contemplated herein
serve to stop the translation of mRNA. They may be either
natural, as described by Kohil, J., Mol. Gen. Genet.
182:430-439 (191); or synthesized, as described th
Pettersson, R.F. Gene 24:15-27 (1983), both of which refer-
ences are specifically incorporated herein by reference.

8.   Selectable Marker

Additionally, it is preferred that the cloning
vector contain a selectable marker, such as a drug resistance
marker of other marker which causes expression of a se-
lectable trait by the host microorganism. In one embodiment
of the present invention, the gene for ampicillin resistance
is included in the vector while in other plasmids, the gene
for tetracycline resistance or the gene for chloramphenicol
resistance is included.

Such a drug resistance or other selectable marker
is intended in part to facilitate in the selection of trans-
formants. Additionally, the presence of such a selectable

marker in the cloning vector may be of use in keeping
contaminating microorganisms from multiplying in the culture
medium. In this embodiment, such a pure culture of the
transformed host microorganisms under conditions would be
obtained by culturing the microorganisms under conditions
which require the induced phenotype for survival.

      B.   <u>Vector Assembly for Microorganisms</u>

Upon synthesis and/or isolation of all necessary
and desired component parts of the above-discussed cloning
vectors, the vectors are assembled by methods generally known
to those of ordinary skill in the art. Assembly of such
vectors is believed to be within the duties and tasks per-
formed by those with ordinary skill inthe art and, as such,
is capable of being performed without undue experimentation.
For example, similar DNA sequences have been ligated into
appropriate cloning vectors, as set forth in Maniatis, T., <u>et
al.</u> <u>Molecular Cloning: A Library Manual</u>, Cold Spring Harbor
Laboratory, N.Y. (1982), which is specifically incorporated
herein by reference.

C.   <u>Host Microorganisms</u>

The vectors and methods disclosed here are suitable
for use in host cells over a wide range of prokaryotic and
eukaryotic organisms.. Prokaryotes are preferred for the
cloning of DNA sequences and for the expression of the gene.
The <u>E. coli</u> strains JM105, JM107 and JM109 (available from
Pharmacis), as well as <u>Bacillus</u> and <u>Pseudomonas</u> species, may
be used for the expression of the gene. In addition to
prokaryotes, eukaryotic microbes such as <u>Saccharomyces
cerevisiae</u> may be used for expression of the gene.

In general, plasmid vectors containing operational
elements which are derived from species compatible with the
hose cells are used. For example, <u>E. coli</u> is typically
transformed using pBR322, a plasmid derived from <u>E. coli</u>.
Table I indicates the list of host organisms and the
competible vectors.

1.   Pseudomonas Vectors

        In addition to the vestors listed in Table I, sev-
eral vector plasmids which autonomously replicate in a broad
range of Gram negative bacteria are preferred for use as
cloning vehicles in hosts of the general Pseudomonas. These
are described by Tait, R.C., Close, T.J., Lundquist, R.C.,
Hagiya, M., Rodriguez, R.L., and Kado, C.I. in Biotechnology,
May, 1983, pp. 269-275; Panopoulos, N.J. in Genetic
Engineering in the Plant Sciences, Praeger Publishers, New
Yoprk, New York, pp. 163-185 (1981); and Sakagucki, K. in
Current Topic in Microbiology and Immunology 96:31-45 (1982),
each of which is specifically incorporated herein by refer-
ence.

        One particular preferred construction would employ
the plasmid RSF1010 and derivatives thereof as described by
Bagdasarian, M., Bagdasarian, M.M., Coleman, S., and timmis,
K.N. in Plasmids of Medical Environmental and Commercial Im-
portance, Timmis, K.N. and Puhler, A. eds., Elsevier/North
holland Biomedical Press (1979), specifically incorporated
herein by reference. The advantages of RSF1010 are that it
is relatively a small, high copy number plasmid which is
readily transformed into and stably maintained in both E.
coli and Pseudomonas species.   In this system, it would be
preferred to use the Tax expression ssytem as described for
Escherichia, since it appears that the E. coli trp promoter
is readily recognized by Pseudomonas RNA polymerase as set
forth by Sakagucki, K. in Current Topics in Microbiology and
Immunology 96:31-45 (1982) and Gray, G.L., McKeown, K.A.,
Jones, A.J.S., Seeburg, P.H., and Heyneker, H.L. in
Biotechnology, Feb. 1984, pp. 161-165, both of which are spe-
cifically incorporated herein by reference. Transcriptional
activity may be further maximized by requiring the exchange
of the promoter with, e.g., an E. coli or P. aeruginosa trp
promoter. Additionally, the lacI gene of a lacI^q strain of
E. coli would also be included in the plasmid to effect regu-
lation.

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Translation may be coupled to translation
initiation for any of the E. coli proteins as described in
the Examples, as well as to initiation sites for any of the
highly expressed proteins of the hose to cause intracellular
expression of the inhibitor.

In those cases where restriction minus strains of a
host Pseudomonas species are not available, transformation
efficiency with plasmid constructs isolated from E. coli are
poor. Therefore, passage of the Pseudomonas cloning vector
through an r- m+ strain of another species prior to transfor-
mation of the desired host, as set forth in Bagdasarian, M.,
et al., Plasmids of Medical, Environmental and Commercial Im-
portance, pp. 411-422, Timmis and Puhler eds., Elsevier/North
holland Biomedical Press (1979), specifically incorporated
herein by reference is desired.

2.   Bacillus Vectors

Furthermore, a preferred expression system in hosts
of the genera Bacillus involves using plasmid pUB110 as the
cloning vehicle. As in other host vectors system, it is pos-
sible in Bacillus to express the IGF binding protein of the
present invention as either an intracellular or a secreted
protein. The present embodiements include both systems.
Shuttle vectors that replicate in both Bacillus and E. coli
are available for constructing and testing various genes as
desired by Dubnau, D., Gryczan, T., Contente, S., and
Shivakumar, A.G. in Genertic Engineering, Vol. 2, Setlow and
hollander eds., Plenum Press, New York, New York, pp. 115-131
(1980), specifically incorporated herein by reference. For
the expression and secretion of the IGF binding protein from
B. subtilis, the signal sequence of alphamalase is preferably
coupled to the coding region for the protein. For snythesis
of intracellular inhibitor, the portable DNA sequence will be
translationally coupled to the ribosome binding site of the
alpha-amylase leader sequence.

Transcription of either of these constructs is
preferably directed by the alph-amylase promoter or a deriva-
tive thereof. This derivative contains the RNA polymerase

recognition sequence of the native alpha-amylase promoter but
incorporates the lac operator region as well. Similar hybrid
promoters constructed from the penicillinase gene promoter
and the lac operator have been shown to function in <u>Bicillus</u>
hosts in a regulatable fashion as set forth by Yansura, D.C.
and Henner in <u>Genetics and Biotechnology of Bacilli</u>, Ganesan,
A.T. and hoch, J.A., eds., Academic Press, pp. 249-263
(1984), specifically incorporated by reference. The lacI
gene of a lacI^q strain of <u>E. coli</u> would also be included in
the placement to effect regulation.

3.   Clostridium Vectors

One preferred construction for expression in
<u>Clostridium</u> is in plasmid pJU12, described by Squires, C.H.
et al., in J. Bacteriol. <u>159</u>:465-471 (1984) and specifically
incorporated herein by reference, transformed into <u>C.
perfringens</u> by the method of Heefner, D.L. et al., as
described in J. Bacteriol. <u>159</u>:460-464 (1984), specifically
incorporated herein by reference. Transcription is directed
by the promoter of the tetracycline resistance gene. Trans-
lation is coupled to the Shine-Dalgarno sequences of this
same tet^r gene in a manner strictly analogous to the proce-
dures outlined above for vectors suitable for use in other
hosts.

4.   Yeast Vectors

Maintenance of foreign DNA introduced into yeast
can be effected in several ways as described by Botstein, D.,
and Davis, R.W., in <u>The Molecular Biology of the Yeast
Saccharomyces</u>, Cold Spring Harbor Laboratory, Strathern,
Jones and Broach, eds., pp. 607-636 (1982), specifically in-
corporated hereby by reference. One preferred expression
system for use with host organisms of the genus <u>Saccharomyces</u>
harbors the IGF binding protein gene on the 2 micron plasmid.
The advantages of the 2 micron circle include relatively high
copy number and stability when introduced into cir° strains.
These vectors preferably incorporate the replication origin
and at least one antibiotic resistance marker from pBR322 to
allow replication and selection in <u>E. coli</u>. In addition, the

plasmid will preferably have two micron sequences and the yeast LEU2 gene to serve the same purposes in LEU2 defective mutants of yeast.

This invention also relates to a recombinant-DNA method for the production of IGF binding protein. Generally, the method includes:

(a) preparation of a portable DNA sequence capable of directing a host microorganism to produce IGF binding protein;

(b) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host microorganism, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into a host microorganism capable of expressing the IGF binding protein;

(d) culturing the host microorganism under conditions appropriate for amplification of the vector and expression of the IGF bining protein; and

(e) in either order:

(i) harvesting the IGF binding protein; and

(ii) causing the IGF binding protein to assume an active tertiary structure.

In this method, the portable DNA sequences are those synthetic or naturally-occurring polynucleotides described above. In a preferred embodiment of the present method, the portable DNA sequence has the nucleotide sequence as defined by the cDNA whose isolation is described in Example 4.

The vectors contemplated as being useful in the present method are those described above. In a preferred embodiment, the cloning vector containing the E. coli origin of replication, a coliphage T7 promoter and a marker for tetracycline resistance is to be used. The gene for IGF

binding protein is translationally coupled to a fragment of
T7 (phi)10 protein.

The vector thus obtained is then transferred into
the appropriate host microorganism, for instance E. coli con-
taining a chromosomal copy of gene 1 from coliphage T7 and
transcribed from the lac promoter. It is believed that any
microorganism having the ability to take up exogeneous DNA
and express those genes and attendant operational elements
may be chosen. It is preferred that the host microorganism
be an anaerobe, facultative anaerobe or aerobe. Particular
hosts which may be preferable for use in this method include
yeasts and bacteria. Specific yeasts include those of the
genus Saccharomyces, and especially Saccharomyces cerevisiae.

Specific bacteria include those of the genera
Bacillus and Escherichia and Pseudomonas. Various other pre-
ferred hosts are set forth in Table I, supra. In other
alternatively preferred embodiments of the present invention,
Bacillus subtilis, Escherichia coli or Pseudomonas aeruginosa
are selected as the host microorganisms.

After a host organism and vector have been chosen,
the vector is transferred into the host organism using meth-
ods generally known by those of ordinary skill in the art.
Examples of such methods may be found in Advanced Bacterial
Genetics by R.W. Davis et al., Cold Spring Harbor Press, Cold
Spring Harbor, New York (1980), which is specifically incor-
porated herein by reference. It is preferred, in one embodi-
ment, that the transformation occur at low temperatures, as
temperature regulation is contemplated as a means of regu-
lating gene expression through the use of operational ele-
ments as set forth below. In another embodiment, if osmolar
regulators have been inserted into the vector, regulation of
the salt concentrations during the transformation would be
required to insure appropriate control of the synthetic
genes.

If it is contemplated that the recombinant IGF
binding protein will ultimately be expressed in yeast, it is
preferred that the cloning vector first be transferred into

<u>Escherichia coli</u>, where the vector would be obtained and
purified after amplification. The vector would then be
transferred into the yeast for ultimate expression of the IGF
binding protein.

The host microorganisms are cultured under condi-
tions appropriate for the expression of IGF binding protein.
These conditions are generally specific for the host organ-
ism, and are readily determined by one of ordinary skill in
the art, in light of the published literature regarding the
growth conditions for such organisms, for example <u>Bergey's</u>
<u>Manual of Determinative Bacteriology</u>, 8th ed., Williams &
Wilkins Company, Baltimore, Maryland, which is specifically
incorporated herein by reference.

Any conditions necessary for the regulation of the
expression of the DNA sequence, dependent upon any operation-
al elements inserted into or present in the vector, would be
in effect at the transformation and culturing stages. In one
embodiment, the cells are grown to a high density in the
presence of appropriate regulatory conditions which inhibit
the expression of the DNA sequence. When optimal cell den-
sity is approached, the environmental conditions are altered
to those appropriate for expression of the protable DNA se-
quence. It is thus contemplated that the production of IGF
binding protein will occur in a time span subsequent to the
growth of the host cells to near optimal density, and that
the resultant IGF binding protein will be harvested at some
time after the regulatory conditions necessary for its
expression were induced.

In a preferred embodiment of the present invention,
the recombinant IGF binding protein is purified subsequent to
harvesting and prior to assumption of its active structure.
This embodiment is preferred as the inventors believe that
recovery of a high yield of re-folded protein is facilitated
if the protein is first purified. However, in one preferred,
alternate embodiment, the IGF binding protein may be allowed
to re-fold to assume its native structure prior to purifica-
tion. In yet another preferred, alternate embodiment, the

IGF binding protein is caused to assume its re-folded state upon recovery from the culturing medium.

In certain circumstances, the IGF binding protein will assure its proper, active structure upon expression in the host microorganism and transport of the protein through the cell wall or membrane or into the periplasmic space. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. The preferred IGF binding protein of the present invention will assume its mature, active form upon translocation out of the inner cell membrane. The structures of numerous signal peptides have been published, for example by Marion E.E. Watson in <u>Nuc. Acid Res.</u>, <u>12</u>:515-5164 (1984), specifically incorporated herein by reference. It is intended that these leader sequences, together with portable DNA, will direct intracellular production of a fusion protein which will be transported through the cell membrane and will have the leader sequence portion cleaved upon release from the cell.

In a preferred embodiment, the signal peptide of the <u>E. colio</u> OmpA protein is used as a leader sequence and is located in a position contiguous with the portable DNA sequence coding for the IGF binding protein structure.

Additionally, preferred leader sequences include those of beta-lactamase, carboxypeptidase G2 and the human signal protein. These and other leader sequences are described.

If the IGF binding protein does not assume its proper structure, any disulfide bonds which have formed and/or any noncovalent interations which have occurred will first be disrupted by denaturing and reducing agents, for example, guanidinium chloride and beta-mercaptoethanol, before the IGF binding protein is allowed to assume its active structure following dilution and oxidation of these agents under controlled conditions.

The transcription terminators contemplated herein serve to stabilize the vector. In particular, those

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sequences as described by Gentz et al,, in Proc. Natl. Acad. Sci. USA 78:4936-4940 (1981) specifically incorporated herein by reference, are contemplated for use in the present invention.

II.   Expression of Genes for IGF binding protein
           in Animal Cells

Methods comparable to the foregoing for the expression of IGF binding protein in higher eukaryotic cells are set forth in Example 6.

The following examples illustrate various presently preferred embodiments of the present invention. The publications provided in these examples are specifically incorporated by reference herein.

EXAMPLES

EXAMPLE 1 - PROTEIN PREPARATION

A.   Materials

Human amniotic fluid was obtained from discarded amniocentesis samples. Ammonium sulfate and sodium chloride were purchased from EM Sciences, Cherry Hill, NJ. DEAE cellulose, ammonium persulfate, sodium thiocyanate, Sephadex G-100, polyethylene glycol (MW 8,000) and ammonium carbonate were purchased from Sigma Chemical Co., St. Louis, MO. Phenyl sepharose CL-4-B was purchased from Pharmacia, Piscataway, NJ. DEAE cellulose was obtained from Whatman and the C-4 reverse phase HPLC column from Vydac, Hesperia, CA. Acetonitrile, Gel Code® silver stain kit and trifloroacetic acid were purchased from Pierce Chemical Co., Rockville, IL. Tris, SDS and TEMED were obtained from Bethesda Research Laboratories, Gaithersburg, MD. Glycine, bromophenol blue, Servalyt® isoelectric focusing Precotes® and glycerol were obtained from Serva, Heidelberg, Germany. Tissue culture plates were purchased from Falcon Labware Division, Becton Dickinson, Oxnard, CA.

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B.   Purification of the Stimulatory
     and Inhibiting IGF Binding Protein
     From Amniotic Fluid

        Crude amniotic fluid (230 ml) was equilibrated with
ammonium sulfate (33% wt/vol) by the addition of 45 gms and
the solution was stirred from 30 min at 8°C. The mixture was
centrifuged 27,000 x g for 20 min then the pellet
reconstituted in 50 cc of 0.05 M Tris HCL pH 7.4. This solu-
tion was adjusted to 50% ammonium sulfate stirred for 30 min
and the centrifugation step repeated. The pellet was
resuspended in 50 cc of 0.05 M Tris pH 7.4 and 1.2 ml of sat-
urated ammonium sulfate added to achieve a final concentra-
tion of 0.14 M. This solution was applied to a phenyl
sepharose column (2.2 x. 15.0 cm) that had been previously
equilibrated with 0.05 M Tris pH 7.4 in 10% ammonium sulfate.
Following sample loading the column was washed with the load-
ing buffer until the absorbance (280 nM) return to baseline.
The column was eluted with step salt gradients containing (1)
0.05 M Tris, pH 7.4, 0.5 sodium thiocyanate, pH 7.4; (2) 0.05
M Tris, pH 7.4; (3) 0.02 M Tris, pH 9.0; and (4) H_2. Each
fraction was assayed for IGF-I binding activity binding
activity as described below in Example I.F. The active frac-
tions were pooled, the pH adjusted to 7.2 with 1.0 M acetic
acid and the solution applied directly to a DEAE cellulose
column that had been equilibrated with 0.01 M $(NH_4)_2$ CO_3,
0.01 M NaCl, pH 7.2. After sample application the column was
washed extensively with the equilibration buffer until the
absorbance (280 nM) returned to baseline. The column was
eluted with step salt gradients containing 0.1, 0.25 and 0.5
and 1.0 M NaCl in the loading buffer. The fractions were
assayed for IGF binding activity as described. Greater than
80% of the activity eluted with 100 or 250 nM NaCl. These
two peaks, termed B and C, were purified separately. 1.5 cc
of the peak B pool was applied to a C-4 Vydac reverse phase
HPLC column (0.46 x 25 cm) that has been equilibrated with
0.04% TFA. The mobile phase was run isocratically for 5 min
then a linear gradient from 0-100% acetonitrile plus 0.04%

TFA was run over 25 min. The IGF binding protein activity of each fraction was determined and the active fractions pooled and stored at -20°C.

Pool C from the ion exchange column was first purified by Sephadex G-100 column chromatography. Ten cc of pool C was applied to a 2.2 x 90 cm column that had been equilibrated with 0.01 (NH₄) CO₃, 0.05 M NaCl, ph 7.2 and approximately 9 cc fractions were collected. The IGF-I binding activity was determined and the active fraction pool applied directly to the reverse phase C-4 column as stated previously were used.

C.   Physicochemical Analyses

The purity of both peaks B and C was determined by SDS polyacrylamide gel electrophoresis. The running gel was 12% acrylamide containing 0.375 M Tris, pH 8.8 and the stacking gel was 4% acrylamide in 0.125 M Tris, pH 6.8. 0.1-10 ug of sample was diluted to 75 ul in 0.1 M Tris, pH 6.8, 10% glycerol, 5% SDS and 0.02% bromophenol blue and the samples were heated to 100°C for 5 min. The supernatants were clarified, the gel lanes loaded, and the proteins separated for 14 hours at 65 volts. Silver staining was performed using Gel code® silver staining kit. The lower limit of detection of the technique was 25 ng as determined using known protein standards. Gels were fixed for 5 hours in 50% ethanol, 5% acetic acid and were then washed with 4 changes of deionized water over a period of 4 hours. The final water wash was replaced by the silver solution (20 ml silver concentrate plus 280 ml water) for 60 minutes with gentle shaking. After a brief H₂O rinse, the reducer solution, containing reducer aldehyde plus reducer base (mixed just prior to use) was added and allowed to gently shake for 8-10 minutes until protein bands of sufficient intensity were observed. The reducer solution was replaced by three successive 30 minute washes with the stabilizer solution containin 20 ml stabilizer base plus 880 ml H₂O.

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D.   Amino Acid Composition and Sequence Analysis

500 ng of each protein was lyophylized under 6N HCl containing 0.1% phenol for one hour at 150°C in vacuo. The amino acids were derivitized with phenylisothiocyanate and the labelled amino acids separated using a NovaPak-C18 Reverse phase. Determination of amino acid sequence is described in Example 3.

E.   Determination of Carbohydrate Content

To determine if either peak B or C contained carbohydrate, 5.0 ug of each peak was loaded on a 12% SDS polyacrylamide gel and separated for 14 hours as described previously. Fetuin was run in parallel as a standard. The gel was fixed 10% acetic acid/25% isopropanol. The gel was then washed sequentially with (1) 0.5% periodic acid; (2) 0.5% sodium arsenite; (3) 0.1% sodium arsenite 5% acetic acid; (4) 5% acetic acid; (5) Schiff's reagent; and (6) 0.6% sodium metabisulfite/0.01 M HCL and stained for 2 hours.

To further determine if either pead B or peak C contained carbohydrate 2.0 ug of each protein was applied to concanavalin-A-sepharose column that had been equilibrated in 0.02 Tris, pH 7.5, 2mM $CaCL_2$ and 2mM $MgCl_2$. The column was slowly loaded over 2 hours and allowed to stand for 45 min at 22°C. The column was further washed with 20 ml starting buffer and then the glycoproteins eluted with 10 ml of 0.02 M Tris, pH 7.5 containing 0.5 M methyl-D-mannoside and 0.1 M NaCl. After one hour the column was eluted. The column was then refilled with the same solution and allowed to stand overnight at 8°C and then eluted. The column was further eluted with 0.02 M Tris, pH 7.5 containing 0.5 M methyl-D-mannoside and 1m NaCl, and the fractions were tested for IGF binding activity as described previously.

F. ^{125}I-IGF-I Binding Capicity

IGF-I binding activity of the column fractions was determined as follows: 10 ul of each fraction was incubated with 40,000 CPM ^{125}I-IGF-I (150 uCi/ug) for 60 minutes at 22°C in 0.1 M Hepes, 0.1% BSA, 0.01% Triton X-100, 44 mM $NaHCO_3$ 0.02%, NaN_3, pH 6.0 (250 ul total volume). The IGF-I

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was iodinated as described by E'Ercole, A.J., Underwood,
L.E., Van Wyk, J.J., Decedue, C.J., and Foushee, D.B. (1976)
in Growth Hormone and Related Peptides (Pecili, A., and
Mueller, E.E., eds.) pp. 190-201, Excerpta Medica, Amsterdam,
specifically incorporated herein by reference. Bound and
free ^{125}I-IGF-I were separated by adding 1% immune serum
globulin and 500 ul of 25% polyethylene glycol (MW 8,000) to
a final concentration of 12.5%. The mixture was centrifuged
at 8,000 x g for 10 minutes then the pellet washed with 6.25%
and the final pellet counted in a gamma spectrometer. Each
pool of active fraction was reassayed at several concentra-
tions and the binding capacity compared to a human amniotic
fluid standard. The data were used to assign a unit value to
each pool. One unit was the quantity of material necessary
to achieve half maximal IGF-I binding.

To determine the binding capacity and affinity of
the pure IGF binding protein for IGF-I, radiolabelled IGF-I
(40,000 cpm/tube) was incubated with 10 ng/ml of each binding
protein and increasing concentrations of unlabelled IGF-I in
0.5 ml of 0.02 M phosphate buffer, pH 7.4. After 48 hours at
4°C the bound and freen ^{125}I-IGF-I were separated by adding a
1:1,000 dilution of a rabbit antibody against the binding
protein and the incubation continued for 12 hours. At that
time, 2 ul of goat antirabbit serum was added and the mixture
incubated for one hour at 22°C then 2 ul or normal rabbit
serum was added and the mixture incubated for an additional
hour. The obund and free growth factor were separated by
centrifugation at 8,000 x g for 10 minutes.

G.   Determination of ^{3}H-Thymidine
      Incorporation into DNA

The biologic activity of pure peak B and C material
was assessed by determining the capacity of each to stimulate
DNA synthesis in porcine aortic smooth muscle cells. The
smooth muscle cells were isolated and maintained in stock
cultures as described by Ross, R. (1971) J. Cell biol. 50,
172-186, specifically incorporated herein by reference. The
cells from stock cultures were subcultured in microtest

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96-well plates (Falcon 3004) by plating at 8,000 cells/well
in DMEN (Gibco) containing 10% fetal bovine serum. Five days
after plating the walls were washed once with serum free DMEM
then test factor added to each well on 0.2 ml DMEM supple-
mented with 1% platelet poor plasma (PPP) and 0.5 uCI
^{3}H-thymidine. PPP was prepared as described by Clemmons,
D.R. (1983) J. Cell Physiol. 114, 61-67, specifically incor-
porated herein by reference. After 36 hours of incubation
the wells are washed twice with Ringer's bicarbonate, twice
with 5% TCA (4°C) then the DNA extracted twice with 0.4 ml of
0.3 N NaOH and ^{3}H-thymidine incorporation determined by liq-
uid scintillation counting.

H.   Isoelectric Focusing

To determine their isoelectric points 2.5 ug of
peak B and C proteins were loaded on to precast isoelectric
focusing plates, pH 3-10, (Servalyt ® Precotes ®), 20 ug of a
known standard was run in a parallel lane. The proteins were
electrofocused for 1 ½ hours at 200 volts then 1 ½ hours at
1000 volts. The gel was divided into two sections and one-
half was fixed 10% TCA then stained with coumassie blue. The
other half was cut into 0.5 cm sections and eluted with 0.04%
TFA. The eluates were analyzed for IGF binding activity as
described previously.

I.   Characteristics of IGF Binding Protein

Ammonium sulfate precipitation of 230 cc of
amniotic fluid resulted in recovery of IGF binding activity
in both the 33 and 50% pellets. The majority of the activity
was present in the 50% pellet and this was chosen for further
purification. During phenyl sepharose chromatography, the
majority of contaminating protein eluted with .05 M sodium
thiocyanate. The peak containing the IGF binding protein
eluted with 0.02 M Tris, pH 9.0 and had been purified 9.5-
fold. Further purification by ion exchange chromatography
resulted in separation of two major peaks of binding activity
which eluted at 100 and 250 mM salt (Fig. 2). These peaks
(termed peaks B and C) were further purified separately.
Peak C material was purified by Sephadex G-100

chromatography. The binding protein activity eluted over a broad peak but was separated from larger molecular weight contaminents (Fig. 3). Sixty ug of G-100 purified material was further purified by reverse phase HPLC using a C-4 column. The active material was eluted as a single peak at 50% acetonitrile and was table during storage in this buffer for periods of up to 3 months (Fig. 4A). Peak B was purified by the same method as C, using the reverse phase HPLC and this step resulted in a 9.4-fold purification (Fig. 4B).

To determine the purity and estimate the molecular size of each protein, the peak C protein at each stage of purification was subjected to polyacrylamide gel electrophoresis under non-reducing conditions followed by silver staining. As shown in Fig. 5 the pure product is 34 Kd and appears as a single bank after the final purification step. The phenyl sepharose step appeared to be the most effective procedure for removing the contaminating proteins. Comparision of peak B and peak C showed that they both had identical Rf values on SDS-PAGE. The molecular for weight estimates were 36 kd under non-reducing conditions but increased to 38 kd if the proteins were reduced prior to electrophoresis. When the amino acid composition of peaks B and C were determined nearly identical amino acid ratios were obtained as set forth in Table II.

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TABLE II

AMINO ACID COMPOSITION OF THE IGF-I BINDING PROTEIN

Amino Acid	Mole %	
	Peak B	Peak C
Aspartic acid and asparagine	7.3	7.6
Glutamic acid and glutamine	15.1	14.9
Serine	8.5	8.7
Glycine	9.0	7.7
Histidine	2.1	2.1
Arginine	4.9	4.6
Threonine	3.8	4.5
Alanine	12.4	11.1
Proline	8.0	8.8
Tyrosine	2.8	2.5
Valine	3.8	3.8
Methionine	0.7	0.6
Cysteine	4.4[a]	5.6[a]
Isoleucine	3.6	3.9
Leucine	8.1	8.2
Phenylalanine	2.0	1.7
Lysine	3.7	3.8
Tryptophan[b]	–	–

    a. Based on recovery of cysteine standard
        following hydrolysis.

    b. Tryptophan is not detectable following
       hydrolysis.

Reduction and alkylation of peak B with amino acid sequencing
showed the following N-terminal sequence showin in Example 3.
Both proteins were stable after heating to 100°C for 10 min.
and were stable to pH 2.5 but IGF binding activity was
destroyed at pH 2.0.

    Further physicochemical analysis was performed to
determine if carbohydrate side chains were present because it
was noted that pure preparations of peak B or C adhered to
concanavalin A. When 2 ug of each protein was applied to the
con A column, 51% of peak C adhered and was eluted with 0.5 M
alphamethylmannoside whereas only 24% of peak B was adherent.
In contrast when 20 ug of either peak B or C was stained with

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Schiff's Base carbohydrate could not be detected in either pro-
tein. Based on the staining intensity of a fetuin standard,
the proteins contained less than 0.8% of their weight as
carbohydrate.

To determine the affinity of each protein for IGF-I
increasing concentrations of unlabelled IGF-I and ^{125}I-IFG were
incubated with peak B or C and the bound complexes immuno-
precipitated. The data were analyzed using Scatchard plots.
Both proteins have binding characteristics that are consistent
with either a two site model with high and low affinity binding
sites or a one site model with negative cooperativity. The
relative affinities of the high affinity sites of the peak B
and C proteins were very similar: 1.06 and 1.1 x 10^{10} L/M re-
spectively.

In spite of their physicochemical similarity with
peak B and C materials were found to have markedly distinct
biologic properties. Pure peak B material greatly potentiated
the smooth muscle cell DNA synthesis response to IGF-I but had
no effect alone or with an equivalent concentration of human
insulin. In contrast, peak C material inhibited basal and
IGF-I stimulated ^{3}H-thymidine incorporation and markedly inhib-
ited the response to peak B plus IGF-I. This effect was
detectable at concentrations as low as 1.0 ng/ml peak C and was
maximal at 20 ng/ml. It appears, therefore that two forms of
the IGF binding protein are present in human amniotic fluid
that have markedly different biologic properties when tested in
a biologic system.

EXAMPLE 2 - POTENTIATION OF IGF-I BY IGF BINDING PROTEIN

1. Cell Culture Techniques

Porcine aortic smooth muscle cells were isolated as
described by Ross (1971) cited above. Stock cultures were
plated at 8,000 cells/cm^2 in 10 cm plastic dishes (Falcon
Labware Division, Becton Dickinson, Oxnard, CA #3001). The
stock cultures maintained in Dulbecco's Modified Eagles Medium
(DMEM) (Grand Island Biological Co., Grand Island, NY) (Gibco)
that was supplemented with 10% fetal bovine serum (FBS)
(Hyclone labs, Ogden, UT). They were passaged every 10-12 days
by removing the cells with trypsin 0.03%, EDTA 0.02% (Gibco)
and replating at a 1:5 dilution. SUBSTITUTE SHEET

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All experiments were conducted using cells between
the 4th and 7th passages. Individual experiments were con-
ducted using cultures that had been plated at 8,000 cells/well
in 96 well microtest plates (Falcon #3004) in 0.2 ml DMEM sup-
plemented with 10% FBS and grown for 5 days. At that time the
serum containing media was removed and test substances were
added to 0.2 ml DMEM supplemented with 1% human platelet poor
plasma (PPP) and 0.5 uCi ^{3}H-thymidine (15 Ci/mmole)
(Schwartz-Mann, Orangeburg, NY). Control wells received DMEM
containing either 1% PPP or varying concentrations of human
serum 1-10%. The PPP and serum were prepared as described by
Clemmons, D.R. (1983) J. Cell Physiol. 114:61-67. The PPP was
determined to have approximately 20 pg/ml platelet factor 4 by
RIA. Following a 36-hour incubation, the reaction was stopped
and the amount of ^{3}H thymidine that had been incorporated into
DNA determined by the method of Clemmons, D.R. (1983) J. Cell
Physiol. 114:61-67.

Chick embryo fibroblasts were isolated from 14-16 day
chicken embryo ski. The primary explants were plated in medium
199 (Gibco) supplemented with 4% FBS (Hyclone), 10% chicken
serum, penicillin 100 U/ml and streptomycin 100 ug/ml (Gibco).
The cells that grew from the explants were passed one time then
subcultured on 96-well plates (Falcon #3004) at 3,000
cells/well in DMEM supplemented with 10% FBS. After five days
the monolayers were washed and test factors added with 0.2 ml
DMEM containing 0.5 uCi ^{3}H-thymidine and 1% PPP then
^{3}H-thymidine incorporation determined after a 36 hour incuba-
tion as described previously. Mouse embryo fibroblasts were
obtained from 18 day fetal Balb/c mouse (skin) and were grown
in DMEM supplemented with 10% FBS, 10mM glutamine (Sigma, St.
Louis, MO) and penicilin 100 U/ml streptomycin 100 mcg/ml
(Gibco). Cultures between the 3rd and 5th passage were used
for all experiments.

Human fibroblasts were purchased from the Human Mu-
tant Genetic Cell Repository (Camden, NJ). They were main-
tained in stock cultures in Minimum Essential Medium (MEM)
(Gibco) supplemented with 1,000 U/ml penicillin, 100 ug/ml
streptomycin and 10% bovine serum (Colorado Serum Co., Denver,

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CO). They were passaged every seven days using a passage dilu-
tion ratio of 1:4. Cultures between the 4th and 5th passages
were plated at a density of 8,000 cells/well on microtest
plates for use in individual experiments. After five days the
cultures were washed twice with MEM then 0.2 ml MEM containing
1% PPP, 0.5 uCi ^{3}H-thymidine and test substances were added
then ^{3}H-thymidine incorporation determined after 36 hours as
described previously.

        To determine if the binding protein could potentiate
the cell growth response to IGF-I, human fibroblasts or smooth
muscle cells were plated at densities of 15,000 cells/cm^2 in 24
well plates (Falcon #3003) containing 1.0 cc DMEM plus 10% FBS
(smooth muscle) or MEM plus 10% calf serum (fibroblasts).
After 2 hours, to allow for cell attachment, the media were
aspirated and 1.0 cc of media containing 1% PPP was added.
After 12 hours incubation to allow the cells to become quies-
cent the media were removed and replaced with 1.0 cc of MEM
containing 1% PPP and the test substances. After 48 hours of
incubation the cultures were exposed to 1.0 cc 0.5% trypsin
0.03% EDTA for 10 min at 37°C. This was removed and added to
9.0 cc of 0.15 mM NaCl and cell number determined using a par-
ticle data counter (Coulter, Model ZBI).

        The binding protein that had been purified from human
amniotic fluid in Example 1 was incubated with quiescent
porcine aortic smooth muscle cell cultures. This cell type was
chosen because it does not have IGF binding protein that is
adherent to its cell surface. Addition of the IGF binding pro-
tein alone resulted in minimal stimulation of ^{3}H-thymidine in-
corporation (Fig. 1A). Addition of concentrations of IGF-I (20
ng/ml) or insulin (10 ug/ml) resulted in only 15 and 38% in-
creases in 3^H thymidine incorporation respectively. In con-
trast, addition of IGF-I (20 ng/ml) plus the pure binding pro-
tein (100 ng/ml) resulted in 4.4 fold stimulation that exceeded
the cellular response to 10% fetal bovine serum. At concentra-
tions of 10 ug/ml, insulin binds to the type I IGF receptor but
does not bind to the binding protein. When these concentra-
tions of insulin were added with the binding protein there was
no potentiation of the cellular replication response indicating

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that activation of the binding protein effect was specific for
IGF.

To determine if cell types from different species
might respond to the IGF binding protein in a similar manner,
the effect of this protein plus IGF-I was tested using chick
and mouse embryo fibroblast cultures. These cell types were
chosen because the chick cells do not synthesize an IGF binding
protein but do possess type I IGF receptors. The mouse cells
secrete a different form of IGF binding protein (MW est. 22K)
and neither cell type has IGF binding protein resulted in only
minimal chances in ^{3}H-thymidine incorporation in either cell
type, (Figs. IB and C). In contrast, 100 ng/ml of the binding
protein plus IGF-I showed marked potentiation of the cellular
response to IGF-I. The chick fibroblasts were particularly
sensitive, reaching 81% of the maximal response induced by 10%
human serum with only 5 ng/ml IGF-I (Fig. IB). The mouse
fibroblasts were also sensitive to the effects of the
coincubation of IGF binding portein and IGF-I (Fig. IC). Nei-
ther cell type responded to insulin plus IGF binding protein
indicating that the binding protein IGF-I complex had to be
formed to achieve the maximal stimulation of ^{3}H-thymidine in-
corporation.

To determine if human fibroblasts, a cell type that
synthesizes the IGF binding protein, could also respond to
exogenously added peptide, quiescent human fibroblast cultures
were prepared as described in Fig. 2. Addition of increasing
concentrations of IGF-I resulted in an increase in ^{3}H-thymidine
update into DNA that was maximal at 20 ng/ml and was 82%
greater than control cultures that were exposed to 1% PPP
alone. The enhanced response to IGF-I alone compared to chick
or mouse fibroblasts may have been due to endogenously secreted
IGF binding protein. Addition of the IGF binding protein alone
resulted in no increase in DNA synthesis. However, when 100
ng/ml of this protein was added with increasing concentrations
of IGF-I DNA synthesis was augumented to a level that was equal
to 10% human serum. The inventors have also determined that
these cells synthesize and secrete the IGF binding protein into
media and that this protein can attach to the cell surface as

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described in Clemmons, D.R.J. Clin. Invest. 77:1548-1556 (1986). However, fibroblasts only secrete sufficient binding protein to reach media concentrations of 2-5 ng/ml and since 5(ng/ml was added to these cultures this difference probably accounts for the additional increase in ^{3}H-thymidine incorporation that was present.

To determine if these responses were dependent upon the concentration of the binding protein, increasing concentrations (1-100 ng/ml) of binding protein plus a constant concentration of IGF-I (10 ng/mI) were incubated with smooth muscle cell cultures. The cultures responded to 2 ng/ml of binding protein with a significant increase in ^{3}H-thymidine incorporation but a maximal effect was not obtained until 100 ng/ml was used (Figs. 2 and 3). Since cultured fibroblasts secrete concentrations of IGF binding protein in the range of 2-5 ng/ml this result indicates that the fibroblasts probably were not secreting a quantity of binding protein sufficient to achieve a maximal DNA synthesis response.

To determine if cells that were stimulated by IGF-I plus the binding protein could traverse the full cell cycle, smooth muscle cells and human fibroblasts were plated at low density and made quiescent by serum deprivation. After 12 hours the binding protein and IGF-I or insulin were added to test cultures and incubated for 60 houses before the cell number was determined. Addition of IGF-I or insulin alone resulted in 3% and 11% increases in smooth muscle cell number, whereas the binding protein plus IGF-I (10 ng/ml) effected a 2.4-fold increase. This increase was greater than that induced by 10% human serum (Fig. 4A). Similar results were obtained with human fibroblasts although the effect of IGF-I alone was greater (Fig. 4B). IGF-I alone resulted in a 31% increase, whereas the combination of binding protein plus IGF-I gave a 2.1-fold increase in cell number after 48 hours.

EXAMPLE 3 - SEQUENCE OF IGF-I BINDING PROTEIN

1. Two methods have been used to determine amino acid sequences from the stimulatory IGFBP purified from amniotic fluid. The protein was reduced in 0.01 M DTT for one hour at 37°C and was alkylated with 0.11 M iodoacetic acid.

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The product was repurified using a reverse phase HPLC column.
In method 1, protein from the reverse phase hplc column was
concentrated and applied directly to the filter of the Applied
biosystems 470A gas phase sequencer. In method 2, protein was
subjected to trypsin digestion (1/50th the amount of protein by
weight) for 12 hours in 0.1 M ammonium carbonate pH 8.0. The
tryptic peptides werre separated by reverse phase hplc on C8
and C4 columns using 0.1% TFA and acetonitrile gradients.
After concentration in a Speedvac the peptides were applied to
the sequences and sequenced as per the manufacturers' instruc-
tions.

The following sequences have been obtained:
1. APWCAPCSAEKLAL (E or S) PPVSASESCVTR (this is the
    N-terminal sequence).
2. WKEPCCIELYR
3. ALPGEQQPLHALTR
4. NGFYHSR
5. FYLPNCNK
6. GQGATVQESDASAP
7. VVESLAK
8. IPGSPEIR
9. ALHVTNIKK

To overlap these peptides, and to obtain other
peptides, the protein should be digested with other proteases
including submaxillary protease (Arg specific), Lys-C protease
(Lys specific), and V8-protease (Glu specific) and the peptides
fractionated by reverse phase hplc and sequenced as described
above.

2.   Amino acid sequence from the purified inhibitory
IGFBP from the amniotic fluid (see purification in Example 1,
above), and the purified stimulatory IGFBPO from the GM10
fibroblast (see purification in Example 5, below), and other
cell-conditioned media can be obtained in ways analogous to
those described above for the stimulatory IGFBP from amniotic
fluid.

EXAMPLE 4 - ISOLATION OF THE GENES FOR IGFBPS
Using the antibodies prepared to the amniotic fluid
stimulatory IGFPB a variety of cell lines have been identified

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that make IGFBPs. These cells include lines GM10 and GM498
(Human Mutant Genetic Cell Repository, Camden, NJ), line MDA
231 (American Type Culture Collection, 123012 Parklawn Dr.,
Rockville, MD 20852-1776) and line HEC-1B (A.T.C.C.). To iso-
late the gene(s) for IGFBPs from these cells they should first
be grown to confluence in 10 150 mm plates in the media pre-
scribed by the supplier and RNA should be isolated from these
cells using the NP-40 lysis procedure (Maniatis, T., Fritsch,
E.F., and Sambrook, J. (1982) Molecular Coloning. A Laboratory
Manual. Cold Spring Harbor Laboratory, pp. 191-193). PolyA$^+$
RNA can be purified by chromatography on oligo dT cellulose
(Aviv. H. and Leder, P. (1972) Proc. Natl. Acad. Sci. (USA) 69,
1408-1412). Five ug of this RNA should be used to synthesize
blunt-ended double-stranded cDNA using the method described by
Gubler and Hoffman (Gubler, U. and Hoffman, B.J. (1983) Gene
25, 263-269). EcoRI linkers should be attached, and the
resulting cDNA cloned into lambda gt11 (Young R.A. and Davis,
R.W. (1983) Proc. Natl. Acad. Sci (USA) 80, 1194-1198.

The resulting library, which should contain upwards
of 10^6 independent clones, may be screened on E. coli Y1090
with an appropriate polyclonal rabbit antibody to stimulatory
IGFBP from amniotic fluid purified as described in Example 1,
using screening conditions described by Young and Davis. Posi-
tive signals will be detected using an alkaline phosphatase
conjugated 2nd antibody (goat anti-rabbit ProMega Biotec) as
described by the manufacturer. Clones testing positive to this
antibody should then be probed with three mixed sequence
oligonucleotide probes based on IGFBP amino acid sequence
(described previously). Probe #1, a 23mer, is a mixture of 192
sequences consisting of all possible DNA sequences for the
amino acid sequence W K E P C C I E. Probe #2, a 17mer, is a
mixture of 64 sequences consisting of all possible DNA se-
quences for the amino acid sequence W Q C A P C. Probe #3, a
17mer, is a mixture of 64 sequences consisting of all possible
DNA sequences for the amino acid sequence P G E Q Q P. These
probes can be synthesized on an Applied Biosystems DNA synthe-
sizer and should be 5' end labeled to a specific activity of
4-6 x 10^6 cpm/pmol using [gamma-32P]-ATP and T4 polynucleotide

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kinase. The clones testing positive to the antibody should be
plated E. coli Y1088 and the DNA transferred to nitrocellulose
as described (Benton, W.D. and Davis, R.W. (1977) Science 196,
180-182).

The probing should be done in a hybridization buffer
containing 1.0M NaCl, 0.1M Sodium Citrate, 2X Denhardts solu-
tion (Denhardt, D.T. (1966) biochem, Biophys. Res. Commun. 23,
641-646), 0.1% SDS, 0.05% Sodium Pyrophosphate and 150 mg/nl
yeast tRNA. The filters should be hydridized for 12-16 hrs.,
using 0.4 pmol/ml of oligonucleotide, at a temperature 2°C
below the calculated Tm for the most AT-rich member of each
pool of oligonucleotides (Suggs, S.V. (1981) In Brown, D.D. and
Fox, C.F. (eds). Developmental Biology Using Purified Genes,
Academic Press, New York, pp. 683-693.) These temperatures are
as follows: Probe #1, 60°C; Probe #2, 50°C; Probe #3, 50°C.
After hybridization, filters should be washed for 45 minutes at
ambient temperature with three changes of 1M NaCl, 0.1M, Sodium
Citrate, and 0.1% SDS. A stringent wash of five minutes should
be done at the calculated Tm (i.e., 2°C above hybridization
temp.) for the most AT rich member of each pool of probes.
Filters should be dried and positive signals detected by
autoradiography. Clones testing positive to the antibody and
both probes should be subcloned into the M13 sequencing vectors
mp 18 and mp 19 (Yanisch-Perron, C., Vierira, J., and Messing,
J. (1985) Gene 33, 103-119 and sequenced as described (Sanger,
F. and Coulson, A.R. (1975) J. Mol. Biol. 94, 441-448).

In the event that no antibody positive clones are
obtained in the first part of the library screening the entire
library should be subjected to the oligonucleotide screening
procedure described above. In addition to the oligonucleotides
described, further obligonucleotides should be used as indi-
cated by protein sequence determined on the purified proteins
(see Examples 1 and 5). The design and synthesis of these
oligonucleotide probes is known to one skilled in the art.

In addition to cell lines, natural human tissues
should be considered as sources of RNA with which to make a
cDNA library. A suitable source of tissue is the decidua of
the uterus.

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A fully length clone will be characterized by its
containing an open reading frame coding for protein sequences
known to be present in IGFBP and start and stop codons. If no
full length clone is isolated one can be assembled from a com-
plete set of partial clones and suitable synthetic DNAs by
methods familiar to one versed in the art. The insert from a
full length clone should be transferred into an expression
vector as described in Example 6.

EXAMPLE 5 - PURIFICATION OF FIBROBLAST IGFBP

Conditioned media from human skin fibroblast cell
lines GM10 or GM498 (Human Mutant Genetic Cell Respository,
Camden, NJ) is centrifuged at 20,000 x g for 20 minutes to re-
move debris. Ammonium sulfate is added to the supernatant to a
final concentration of 65% and the solution allowed to stir
slowly at 4°C for 30 min. The pellet from a 20,000 x g
centriguation for 30 min. is extracted with 10 mM ammonium car-
bonate pH 7.2 containing 0.5 M NaCl. The extracts are frac-
tioned by chromatography on Sephadex G-100 equilibrated in the
extraction buffer. Fractions containing IGF-I binding activity
are pooled and reprecipitated with 65% ammonium sulfate.

The pellet from a 20,000 x g centrifugation for 30
min. is extracted with 10 mM ammonium carbonate pH 6.5 and
diluted with distilled water to a conductivity equal to that of
50 mM NaCl. The solution is applied at a rate of 15 ml/hour to
a heparin sepharose column previously equilibrated with 10 mM
ammonium carbonate pH 6.5 and 50 mM NaCl. The column is washed
with three volumes of the equilibration buffer containing 1 M
NaCl and then three volumes of buffer containing 2 M NaCl. The
column containing buffer with 2 M NaCl is alloed to stand at
4°C overnight and in the morning is eluted once more with one
volume of 2 M NaCl. The pooled 2 M NaCl washes are pooled and
reverse dialysed against 10 mM ammonium carbonate pH 6.5 and 50
mM Nacl.

The dialysate is applied at 15 ml/hour to an IGF-I
affinity column, prepared from 2 mg of IGF-I and 3 ml of
Reactigel X (Pierce Chemical) according to the manufacturers'
instructions, which was preequilibrated with 10 mM ammonium
carbonate pH 6.5 50 mM NaCl. The column is washed with five

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volumes of equilibration buffer and then with five volumes of
10 mM ammonium acetate pH 4.5 50 mM NaCl. The IGFBP activity
is then eluted with 1 M acetic acid.

        The active fractions are applied to a Vydac C-4 re-
verse phase hplc column and eluted with 0.04% aqueous TFA for 5
min. at 1 ml/min followed by a linear gradient of acetonitrile
to 25% over a period of 3 min. The protein is then eluted with
a linear gradient of from 25% to 100% acetonitrile over a peri-
od of 30 min., eluting close to 30% acetonitrile. If insuffi-
ciently pure for sequencing at this state the protein is
diluted in water and reapplied to a Vydac C-8 column. It is
eluted with the same gradient applied to the C-4 column,
eluting at about 50% acetonitrile.

EXAMPLE 6 - EXPRESSION OF GENES FOR IGFBP IN ANIMAL CELLS

        To express IGFBP in animal cells the following steps
should be carried out:

        1. Construction of an expression vector
        2. Choice of a host cell line
        3. Introduction of the expression vector into the
cells
        4. Introduction of expression of IGFBP.

        1. To construct an IGFBP expression vector that will
function in many different cells types a plasmid should be con-
structed as follows. pBR322 should be digested with EcoRl and
EcoRV and the EcoRl sticky end should be blunt-ended by treat-
ment with Klenow DNA polymerase in the presence of dATP, and
dTTP. The large fragment should be isolated and ligated to the
4 kb EcoRl fragment of pMK. (Brinster et al. Cell 27 223-231,
1981) carrying the MT1 promoter and the structural gene for
herpes simplex type 1 thymidine kinase (HSV-1 tk), blunt ended
as described above. The ligation mixture should be transformed
into E. coli JM109 and plasmid DNA prepared from ampicillin re-
sistant, tetracycline sensitive transformants. The DNA should
be characterized by restriction mapping and a plasmid con-
forming to the structure drawn in Fig. 5 selected for further
work.

        The Bgl II site of this plasmid should be converted
to an EcoRl site by digesting the DNA with Bgl II and cloning

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into the linearised DNA a suitable piece of synthetic DNA con-
taining an EcoRl site using standard methods. The resulting
plasmid, pMK-SGE should be amplified in E.coli, reisolated by
standard methods, and linearised with EcoRl.

Full length IGFBP cDNA isolated from DNA of lambda
gtll clones by standard methods that include digestion of the
DNA with EcoRl and gel purification should be ligated into the
EcoRl site of the MT-1 promoter plasmid pMK-SGe described
above. Following this ligation and transformation of E coli, a
clone should be selected in which the IGFBP cDNA is in the same
orientation as the MT-1 promoter as determined by restriction
mapping. Plasmid DNA snould be prepared for transformation of
animal cells.

2. Because the active IGFBPs may have as yet
uncharacterized postranslational modifications the genes should
be expressed in cells as like as possible to their natural cell
of origin. The procedure to be followed is illustrated here
using the gene for the stimulatory IGFBP from fibroblasts.
Similar procedures should be followed with genes for the other
proteins once suitable producer cell lines have been identi-
fied. Until that time fibroblasts will act as producer cells
for those IGFBPs as well.

3. To introduce the expression vector into Ltk-cells
or other TK⁻ fibroblast lines, the expression vector should be
mixed with pHSV-106 plasmid DNA (Bethesda Research Labs) which
carries the HSV-1 TK gene. The mixed DNAs should be applied to
the cells using the standard calcium phosphate-DNA precipita-
tion techniques. S. L. Graham and A. J. Von der Eb (1973)
Virology Vol. 52:456-467. Stable transformants can be selected
by their ability to grow in medium containing hypoxanthine,
aminopterin and thymidine M. Wigler et al. (1979) Cell
16:777-785.

To introduce the IGFBP expression vector DNA into
fibroblasts that are not TK⁻ it should be mixed with a selec-
tion vector prepared by fusing the SV40 promotor in pKSV-10
with the neomycin resistance (aminoglycoside
3'-phosphotransferase) gene (Pharmacia) and cotransformed into
the cells as described above. Stable transformants can be

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selected by their ability to grow in the presence of the antibiotic G418.

A third method for introducing the expression vector into mammalian cells is to cotransform the expression vector with a plasmid carrying a functional dihydrofolate reductase (DHFR) gene. Stable transformants can be selected by growing the cells in methotrexate. This method is particularly suitable for stable transfaction of DHFR⁻ CHO cells F. McCormick et al. (1984) Mol. Cell. Biol. 4:166-172..

To induce expression of the IGFBP genes in each of the above stably transformed animal cells, the cells should be exposed to 10 mM cadmium sulfate in the growth medium. The MT-1 promoter is induced by this treatment and should result in an increase in expression of the IGFBP gene of approximately 7 fold. (Mayo, K.E., R. Warren and R.D. POalmiter 1982 Cell 29:99-108).

To increase expression of the IGFBP further the copy number of the IGF-I stimulator DNA should be amplified by one of the following methods. In the case where the expression vector was contransformed with the TK gene, amplification should be brought about by decreasing the thymidine concentration in the medium or omitting it entirely. In the case in which the expression vector was introduced into cells by cotransformation with the DHFR gene, amplification of the gene should be brought about by increasing concentration of methotrexate in the medium. (F. McCormick et al. (1984) Mol. Cell. Biol 4:166-172.

EXAMPLE 7 PURIFICATION OF IGFBPS FROM RECOMBINANT ANIMAL CELLS

Since the IGFBPs are expected to be secreted from cells like the natural material it is anticipated that the methods described above the purification of the natural protein will allow similar purification and characterization of the recombinant protein.

EXAMPLE 8

A lambda gt-11 human uterus decidua cDNA library was created as described previously and screened with a rabbit polyclonal antibody to stimulatory insulin-like growth factor binding protein (IGFBP) by the method of Young and Davis as

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described in Example 4. The rabbit polyclonal antibody was
that described in Example 1. Positive signals were detected
using an alkaline phosphate conjugated second antibody (goat-
anti-rabbit, Pro Mega Biotec) as described by the manufacturer.
Approximately 0.12% of the clones in the library tested posi-
tive to the antibody. Twleve independent clones were purified
and the inserts compared by agarose gel electrophoresis. A
clone with an insert of approximately 1500 BP was subcloned
into the M13 sequencing vector mp 18 in both orientations and
the sequence was determined by the method of Sanger et al.
This clone contained a single open reading frame of 776 BP
encoding a 24 amino acid signal sequence followed by a 233
amino acid polypeptide that included all peptide sequences pre-
viously described.

The sequence has been determined and is set forht in
Figure 6.
This sequence contains a tripeptide ArgGlyAsp. This tripeptide
or peptides which contain this tripeptide and flanking residues
from the IGFBP sequence may serve to prevent cell or matrix
attachment of IGFBP and thereby prevent its stimulatory effect
on IGF-I induced cell growth.

Example 9

EXPRESSION OF THE IGFBP GENE IN PROCARYOTIC

(E. COLI) CELLS AND THE ISOLATION OF

BIOLOGICALLLY ACTIVE IGFBP FROM THEM

A.   Preparation of Expression Vectors for IGFBP

The IGFBP cDNA has been cloned into the E. coli, T7
RNA polymerase-dependent expression vector pJU1003 by the fol-
lowing steps are illustrated in Figure 7.

1.   Plasmid pJU1003 was digested with the restric-
       tion enzymes BamHI and EcoRI and the large lin-
       ear vector fragment purified by standard gel
       methods.

2.   The lambda gt11 clone containing the IGFBP cDNA
       coding sequence was digested with the enzymes
       EcoRI and BstEll, releasing a fragment of 1225

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bp that contains the mature IGFBP coding se-
quence from codon 26 through the C-terminus and
an additional 600 bp of 3' untranslated se-
quence. This fragment was also purified by
standard gel methods.

3.   The synthetic double-stranded oligonucleotide of
102 bp shown in Figure 7 coding for the first 25
amino acids of IGFBP and an N-terminal
methionine (ATG) codon was prepared. The codons
chosen reflect E. coli's preference for certain
codons in highly expressed genes. In addition,
the 5' end of this oligonucleotide contains DNA
sequences that result in translational coupling
of the vector-encoded T7 010 gene as described
by Rosenberg, A.H., Lade, B.N., Chui, D., Lin,
S., Dunn, J.J., Studier, F.W., in Gene
56:125-135, 1987, specifically incorporated
herein by reference, to the IGFBP gene. The
oligonucleotide has BamHI and BstEll cohesive
ends.

4.   The three DNA sequences described above were
ligated together, and that mixture was used to
transform E. coli strain BL21/DE3 described by
Studier, F.W., and Moffatt, B., in J. Mol. Biol.
189:113-130, 1986, specifically incorporated
herein by reference. Colonies resistant to
15 ug/ml tetracycline were selected and screened
for production of IGFBP following induction with
1 mM isopropyl B-D-thiogalactopyranoside (IPTG)
using the anti-IGFBP antibody as described in
Example 4 above.

5.   Plasmid DNA was prepared from a positive clone,
and that part resulting from the synthetic
oligonucleotide was sequenced according to the
method of Sanger and Coulson described in J.
Mol. Biol. 94:441-448, 1975, specifically incor-
porated herein by reference.

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A transformant called pJU1020 was isolated as
described above and was grown up in liquid media. The amount
of IGFBP produced following induction with 1 mM IPTG was esti-
mated by SDS PAGE of total cellular protein using purified na-
tive (amniotic fluid) IFGBP as the standard. It was estimated
that approximately 1 ug of IGFBP/ml of culture/A_{600} unit was
produced. Importantly, this material migrated in the gel with
the same apparent molecular weight as the native protein,
31 kD. This observation shows by this criterion that material
identical to the native IGFBP can be produced in E. coli.

To make a more efficient producer of IGFBP, the 3'
untranslated portion of the cDNA clone was removed from plasmid
pJU1020 in the following way as shown in Figure 8.

1.   The plasmid was digested with the restriction
      enzymes BamHI and HindIII.

2.   The synthetic double-stranded oligonucleotide of
      74 bp shown in Figure 8 was prepared. This
      oligonucleotide comprises (a) the coding se-
      quence for the C-terminal 21 codons of IGFBP
      using codons favored by E. coli; (b) a transla-
      tion termination codon for IGFBP; (c) additional
      translation termination codons in the other
      translation reading frames; (d) terminal HindIII
      and BamHI restriction sites.

3.   The synthetic oligonucleotide is then mixed with
      the digested DNA described in (1) and ligated
      together. After ligation, the mixture was
      digested with the enzyme EcoRI. This procedure
      linearizes only those molecules containing the
      3' untranslated DNA since this site is unique to
      that DNA in this experiment.

4.   This mixture was then used to transform E. coli
      strain BL21/DE3 with selection for tetracycline-
      resistant (15 ug/ml) clones. Transformants were
      screened for IGFBP production and correctness of
      the DNA sequence of the synthetic
      oligonucleotide portion of the clone as
      described in connection with the pJU1020
      construction above.   SUBSTITUTE SHEET

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A positive and correct clone (pJU1021) was grown and
the production of IGFBP following induction of gene expression
measured as described with the pJU1021 construction described
above. This clone produces approximate 20 ug of IGFBP/ul of
culture/A$_{600}$ unit.

The T7 gene 10 coupled IGFBP sequence described above
was transferred to a plasmid vector containing the tacI promot-
er as described by deBoer, H.A., Comstock, L.J. and Vassar, M.,
in Proc. Natl. Acad. Sci. USA $\underline{80}$21-25, 1983, specifically in-
corporated herein by reference, by the following steps as set
forth in Figure 9.

1. Plasmid pT3XI-2, an E. coli expression plasmid
    containing the tac promoter, was digested with
    the enzymes EcoRI and HindIII and the large
    fragment containing the promoter, antibiotic re-
    sistance genes and origin of replication was
    isolated.

2. Plasmid pJU1021 was digested with the enzymes
    XbaI and HindIII and the fragment containing the
    IGFBP coding sequence isolated. This fragment
    also contains the T7 gene 10 sequences and the
    translational coupler as described in connection
    with the pJU1021 construction described above.

3. A synthetic oligonucleotide 20 bp in length was
    prepared. This fragment contains the DNA se-
    quence just upstream (5') to the XbaI site of
    plasmid pJU1021. This DNA is used to adapt the
    IGFBP coding sequence to the tac promoter con-
    taining expression plasmid.

4. These three DNA sequences were ligated together
    and the mixture used to transform E. coli strain
    JM107 with tetracycline-resistant clones se-
    lected. Clones expressing IGFBP following gene
    induction with 1 mM IPTG and containing the cor-
    rect DNA sequence of the synthetic
    oligonucleotide were identified as described
    above. A positive and correct clone was called
    pJU1022. This clone was grown and the

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production of IGFBP following induction of gene expression was measured as described with the pJU1020 construction. This clone produces approximately 10 ug of IGFBP/ml of culture /A_{600} unit.

B.   Production of IGFBP from E. coli

Preparation of biologically active IGFBP from E. coli was done in the following way. E. coli strain BL21/DE3 containing plasmid pJU1021 was grown at 37°C in Luria broth containing 15 ug/ml of tetracycline to an A_{600} of 1.0. One mM IPTG was then added to induce IGFBP gene expression, and the growth continued for 2 hours. Then the cells were harvested by centrifugation and resuspended in one-twentieth their original culture volume in 50 mM Tris, pH 7.5, and lysed in a French pressure cell. Following centrifugation at 15,000 x g for 10 minutes, the pellet (insoluble protein) and supernatant (soluble protein) were analyzed by SDS-PAGE for the presence of IGFBP, Western blotting using rabbit anti-IGFBP antibodies. Approximately 80% of the total IGFBP was found in the insoluble fraction.

Biologically active IGFBP has been obtained from this function by three different methods.

1.   The pellet can be washed with 50 mM Tris, pH 7.5 (1/20 original culture volume. Approximately 5% of the IGFBP will then solubilize and remain in the supernatant following centrifugation. This material contains monomeric, dimeric and higher molecular weight variants of IGFBP and has been shown to potentiate the activity of IGF-I in the porcine aortic smooth muscle cell bioassay described above.

2.   The insoluble protein pellet prepared as described above can be resuspended in 6 M Guanidine HCL (1 ml/50 ml of original cell culture), and left at room temperature for 5 minutes. Dithiothreitol is added to a final concentration of 20 mM, and the mixture left at room temperature for 30 minutes. This mixture is centrifuged at 13,000 xg for 15 minutes and the supernatant is mixed with oxidized glutathione to a final concentration of 20 mM and the mixture left at room temperature for 10 minutes.

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4.5 ml of 50 mM tris pH 10.7 per 50 ml of original culture and cysteine to 1 mM final contration are added to the mixture and it is left at room temperature overnight. The mixture is then dialyzed against 50 mM tris pH 7.5 and centrifuged at 13,000 xg for 15 minutes to remove the insoluble materia.

The IGFBP obtained from this procedure is called "refolded IGFBP". When it is analyzed by SDS-PAGE without prior reduction, it can be shown to contain a dimetric form of IGFBP (apparent molecular weight 50 kDa) as well as the monomeric form (apparent molecular weight 25 kDa). Both of these forms bind IGF-I as shown by an $\underline{in}$ $\underline{vitro}$ assay showing the binding of ^{125}I-labelled IGF-I to IGFBP transferred to a nitro-cellulose filter following non-reducing SDS-PAGE. The refolded material potentiates the activity of IGF-I in the mitogenicity assay using porcine aortic smooth muscle cells as described.

Monomeric IFGBP can be purified free of dimeric by FPLC (Mono Q) with the monomers eluting at 0.28 M NaCl. This fraction containing only monomers does not potentiate the activity of IGF-I in the mitogenicity assay using porcine aortic smooth muscle cells as described.

3.   The insoluble protein pellet can be solubilized with 6 M guanidine hydrochloride as above but without the addition of dithiothreitol and centrifuged. If this material is then dialyzed against 50 mM tris pH 7.5, a precipitate forms. However, if this precipitate and the soluble protein are compared by SDS-PAGE, it is found that at least 50% of the IGFBP solubilized by 6 M Guanidine HCL remains in solution following dialysis. This solubilized material can be shown to contain monomeric and dimeric forms of IGFBP that bind IGF-I as described above. This guanidine solubilized and dialyzed IGFBP has also been shown to potentiate the activity of IGF-I in the mitogenicity assay using porcine aortic smooth muscle cells as described.

It will be apparent to those skilled in the art that various modifications and variations can be made in the processes and products of the present invention. Thus, it is intended that the present invention cover the modifications and

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variations of this invention provided they come within the
scope of the appended claims and their equivalents.

WHAT IS CLAIMED IS:

1.   An insulin like growth factor binding protein capable of inhibiting the activity of insulin like growth factor.

2.   An insulin like growth factor binding protein capable of potentiating the activity of insulin like growth factor.

3.   An insulin like growth factor binding protein having the amino acid sequence set forth in Figure 6.

4.   A vector containing a DNA sequence coding for an insulin like growth factor binding protein.

5.   The vector of claim 4 wherein the vector contains the DNA sequence set forth in Figure 6.

6.   A recombinant-DNA method for the production of insulin like growth factor binding protein comprising:

(a)   Transforming a unicellular host with the vector of claim 4 wherein said vector also contains an origin of replication appropriate for said unicellular host;

(b)   Culturing the transformed host under conditions appropriate for the production of insulin like growth factor binding protein.

7.   The method of claim 6 wherein said unicellular host is E. coli.

8.   The method of claim 6 wherein said vector contains the DNA sequence of Figure 6.

9.   The method of claim 6 wherein said vector is pJU1020.

10.   The method of claim 6 wherein said vector is pJU1021.

11.   The method of claim 6 wherein said vector is pJU1022.

12.   The vector pJU1020.

13.   The vector pJU1021.

14.   The vector pJU1022.

15.   An insulin like growth factor binding protein produced by the method of claim 6.

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

EcoRI

EcoRV

pBR322

DIGEST WITH
EcoRI AND
EcoRV.

FILL IN EcoRI
STICKY END TO
FORM BLUNT END.

→ 1.

BLUNT
END

BLUNT
END

EcoRI   MT-1
         PROMOTER

BglII

pMK

Hsv-1
TK

EcoR

DIGEST WITH EcoRI
FILL IN ENDS TO
FORM BLUNT ENDS.

PURIFY 4Kb FRAGMENT
CONTAINING MT-1 PROMOTER,
BglII SITE AND
Hsv-1 TK.

→ 2.

BLUNT
END

MT-1 PROMO-
TER

BglII

Hsv-1
TK

BLUNT END

MIX FRAGMENTS 1 AND 2.
LIGATE, TRANSFORM E. Coli
ISOLATE PLASMID DNA.
CHECK STRUCTURE BY
RESTRICTION DIGESTS.

THE PLASMID SHOULD
BE 8Kb IN LENGTH WITH
NO EcoRI SITES AND ONE
BglII SITE.

pMK-SGE

MT-1
PROMOTER

BglII

Hsv-1
TK

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FIG. 6

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```
         10          20          30          40          50          60
CCGCCGCCACCCTCCCAGAGAGCACTGGCCACCGCTCCACCATCACTTGCCCAGAGTTTG


         70          80          90         100         110         120
GGCCACCGCCCGCCGCCACCAGCCCAGAGAGCATCGGCCCCTGTCTGCTGCTCGCGCCTG


        130         140         150        . 160         170         180
GAGATGTCAGAGGTCCCCGTTGCTCGCGTCTGGCTGGTACTGCTCCTGCTGACTGTCCAG
     MetSerGluValProValAlaArgValTrpLeuValLeuLeuLeuThrValGln


        190         200         210         220         230         240
GTCGGCGTGACAGCCGGCGCTCCGTGGCAGTGCGCGCCCTGCTCCGCCGAGAAGCTCGCG
ValGlyValThrAlaGlyAlaProTrpGlnCysAlaProCysSerAlaGluLysLeuAla


        250         260         270         280         290         300
CTCTGCCCGCCGGTGTCCGCCTCGTGCTCGGAGGTCACCCGGTCGCCGGCTGCGGCTGTT
LeuCysProProValSerAlaSerCysSerGluValThrArgSerProAlaAlaAlaVal


        310         320         330         340         350         360
GCCCGATGTGCGCCCTGCCTCTGGGCGCCGCGTGGCGTGGCGACTGCACGCTGCGCCCGG
AlaArgCysAlaProCysLeuTrpAlaProArgGlyValAlaThrAlaArgCysAlaArg


        370         380         390         400         410         420
GGACTCAGTTGCCGCGCGCTGCCGGGGGAGCAGCAACCTCTGCACGCCCTCACCCGCGGC
GlyLeuSerCysArgAlaLeuProGlyGluGlnGlnProLeuHisAlaLeuThrArgGly


        430         440         450         460         470         480
CAAGGCGCCTGCGTGCAGGAGTCTGACGCCTCCGCTCCCCATGCTGCAGAGGCAGGGAGC
GlnGlyAlaCysValGlnGluSerAspAlaSerAlaProHisAlaAlaGluAlaGlySer


        490         500         510         520         530         540
CCTGAAAGCCCAGAGAGCACGGAGATAACTGAGGAGGAGCTCCTGGATAATTTCCATCTG
ProGluSerProGluSerThrGluIleThrGluGluGluLeuLeuAspAsnPheHisLeu


        550         560         570         580         590         600
ATGGCCCCTTCTGAAGAGGATCATTCCATCCTTTGGGACGCCATCAGTACCTATGATGGC
MetAlaProSerGluGluAspHisSerIleLeuTrpAspAlaIleSerThrTyrAspGly


        610         620         630         640         650         660
TCGAAGGCTCTCCATGTCACCAACATCAAAAAATGGAAGGAGCCCTGCCGAATAGAACTC
SerLysAlaLeuHisValThrAsnIleLysLysTrpLysGluProCysArgIleGluLeu


        670         680         690         700         710         720
TACAGAGTCGTAGAGAGTTTAGCCAAGGCACAGGAGACATCAGGAGAAGAAATTTCCAAA
TyrArgValValGluSerLeuAlaLysAlaGlnGluThrSerGlyGluGluIleSerLys


        730         740         750         760         770         780
TTTTACCTGCCAAACTGCAACAAGAATGGATTTTATCACAGCAGACAGTGTGAGACATCC
PheTyrLeuProAsnCysAsnLysAsnGlyPheTyrHisSerArgGlnCysGluThrSer


        790         800         810         820         830         840
ATGGATGGAGAGGCGGGACTCTGCTGGTGCGTCTACCCTTGGAATGGGAAGAGGATCCCT
MetAspGlyGluAlaGlyLeuCysTrpCysValTyrProTrpAsnGlyLysArgIlePro


        850         860         870         880         890         900
GGGTCTCCAGAGATCAGGGGAGACCCCAACTGCCAGATATATTTTAATGTACAAAACTGA
GlySerProGluIleArgGlyAspProAsnCysGlnIleTyrPheAsnValGlnAsnEnd
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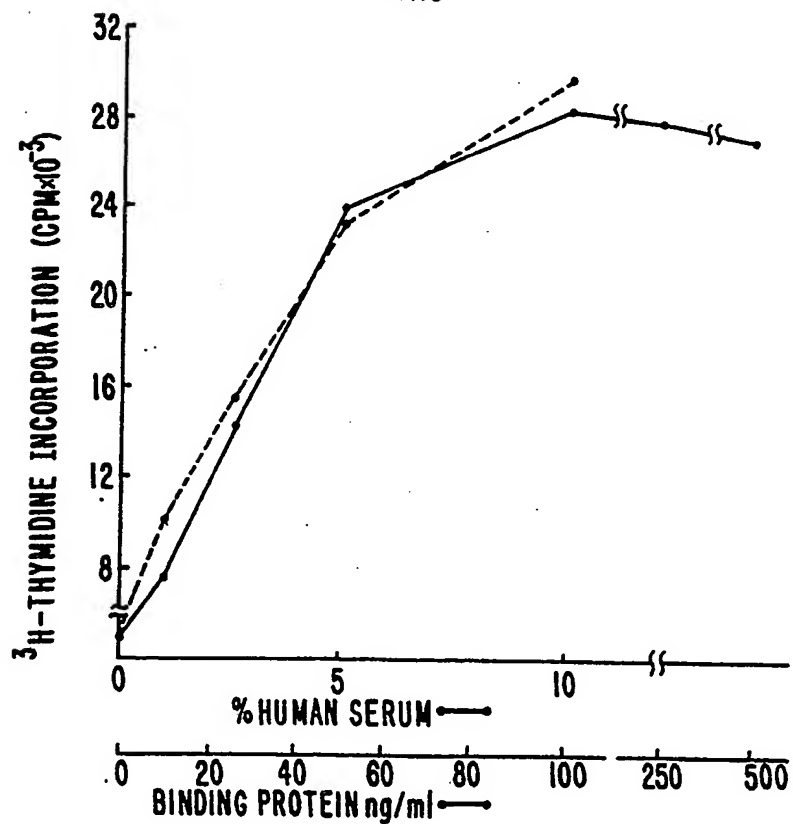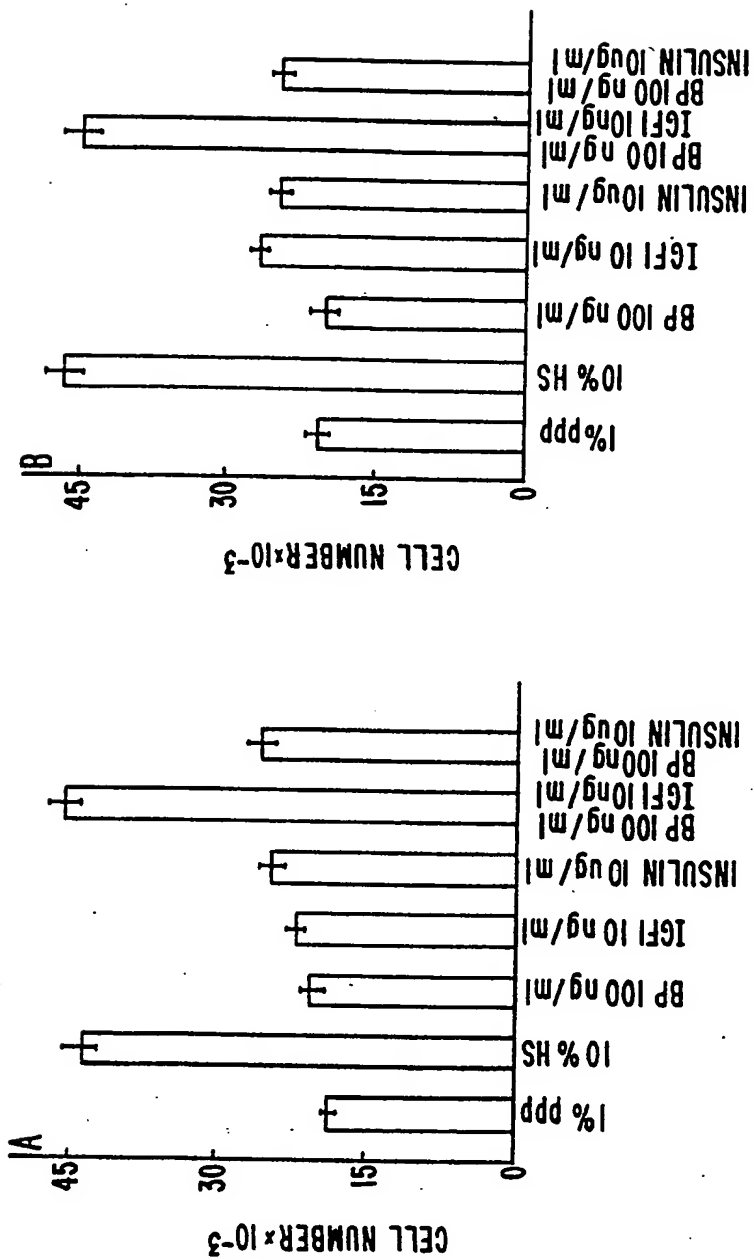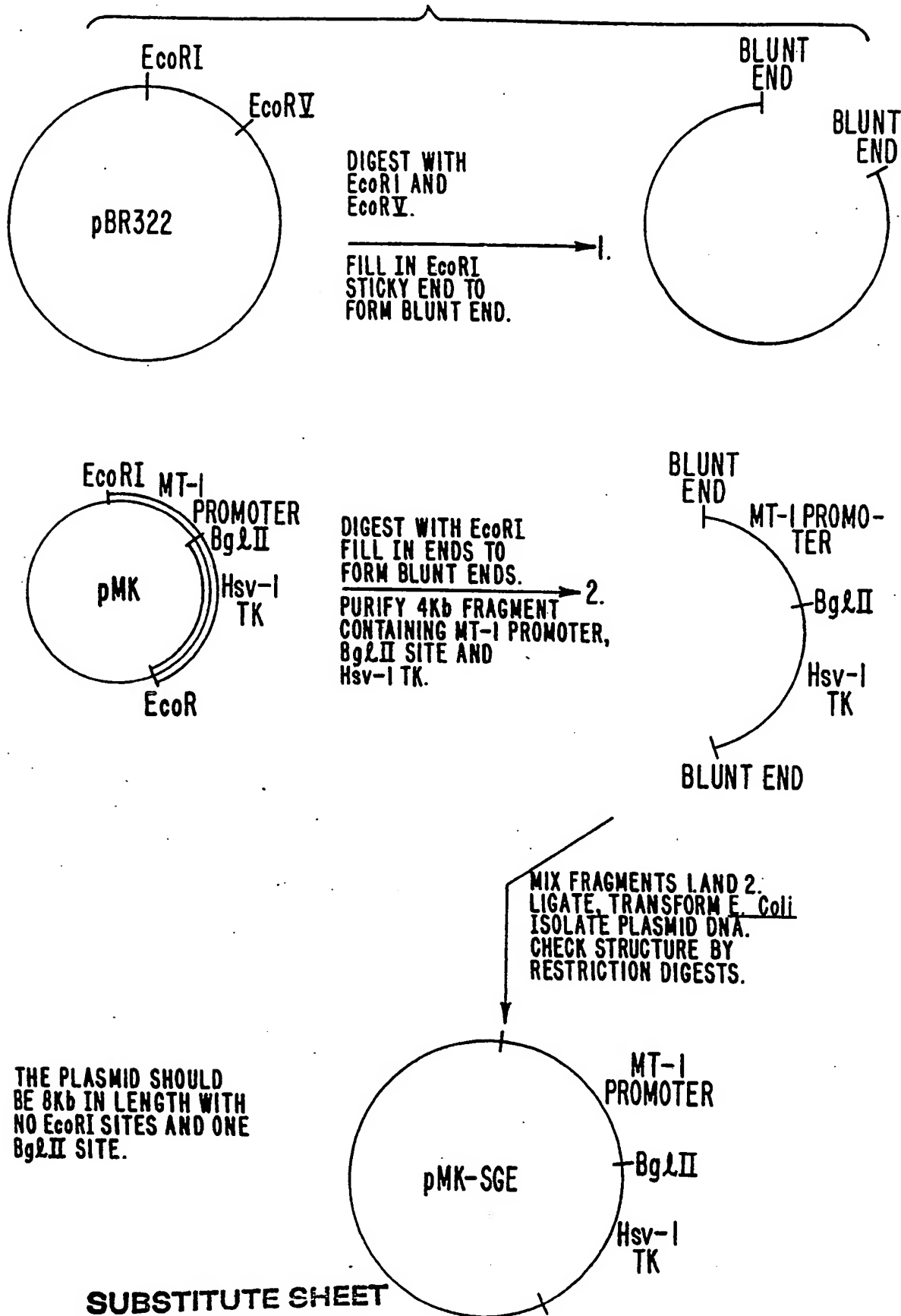
FIG. 6 (cont.)

```
         910        920        930        940        950        960
AACCAGATGAAATAATGTTCTGTCACGTGAAATATTTAAGTATATAGTATATTTATACTC

         970        980        990       1000       1010       1020
TAGAACATGCACATTTATATATATATGTATATGTATATATATAGTAACTACTTTTTAT

        1030       1040       1050       1060       1070       1080
ACTCCATACATAACTTGATATAGAAAGCTGTTTATTTATTCACTGTAAGTTTATTTTTTC

        1090       1100       1110       1120       1130       1140
TACACAGTAAAAACTTGTACTATGTTAATAACTTGTCCTATGTCAATTTGTATATCATGA

        1150       1160       1170       1180       1190       1200
AACACTTCTCATCATATTGTATGTAAGTAATTGCATTTCTGCTCTTCCAAAGCTCCTGCG

        1210       1220       1230       1240       1250       1260
TCTGTTTTAAAGAGCATGGAAAAATACTGCCTAGAAAATGAAAATGAAATAAGAGAGAGT

        1270       1280       1290       1300       1310       1320
AGTTTTTCAGCTAGTTTGAAGGAGGACGGTTAACTTGTATATTCCACCATTACATTTGAT

        1330       1340       1350       1360       1370       1380
GTACATGTGTAGGGAAAGTTAAAAGTGTTGATTACATAATAAAGCTACCTGTGGTGATGT

        1390       1400       1410       1420       1430       1440
TGCCACCTGTTAAAATGTACACTGGATATGTTGTTAAACACGTGTCTATAATGGAAACAT

        1450       1460       1470
TTACAATAAATATTCTCGATGGAAAAAAAAAAAAAAAA
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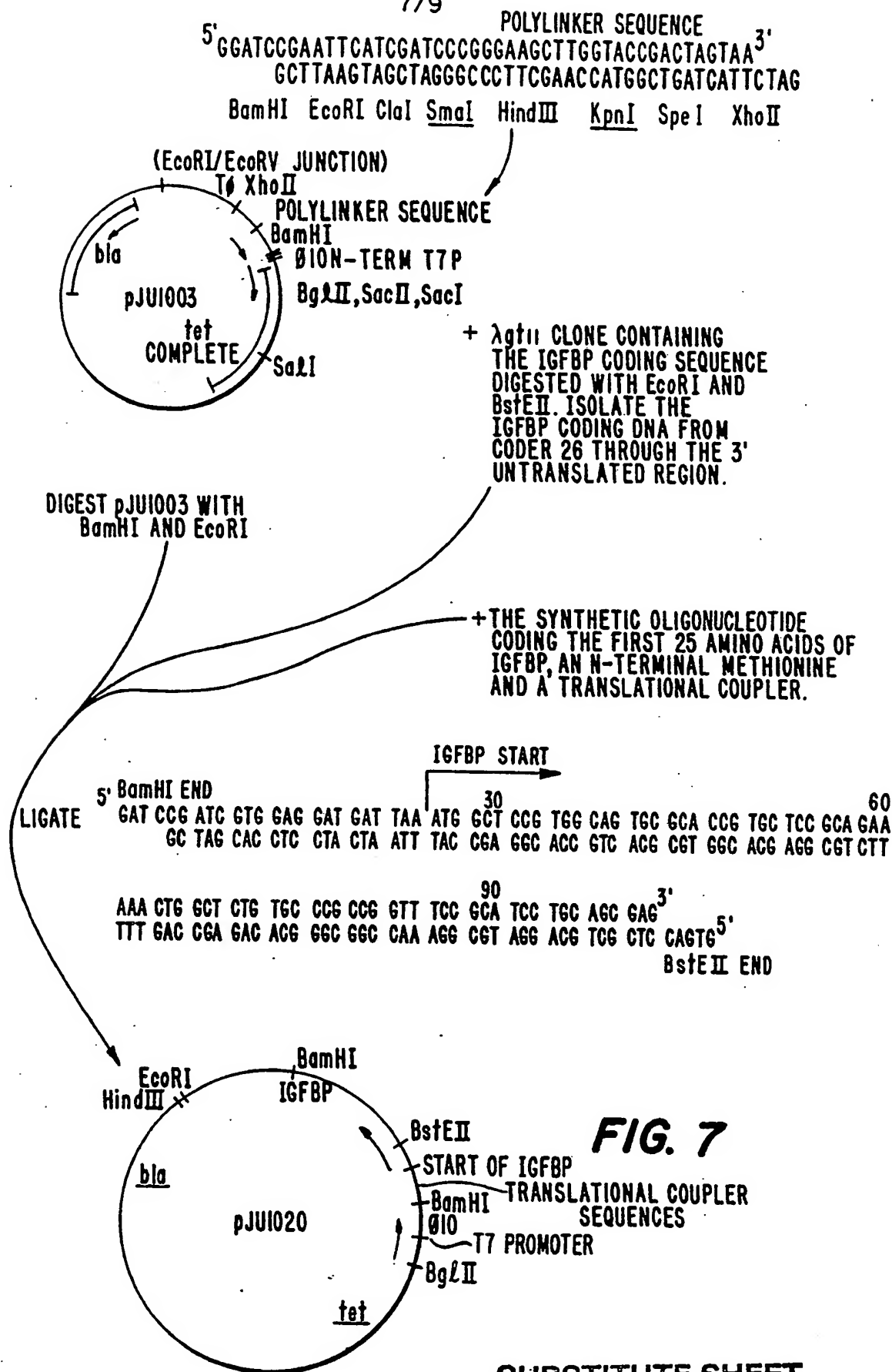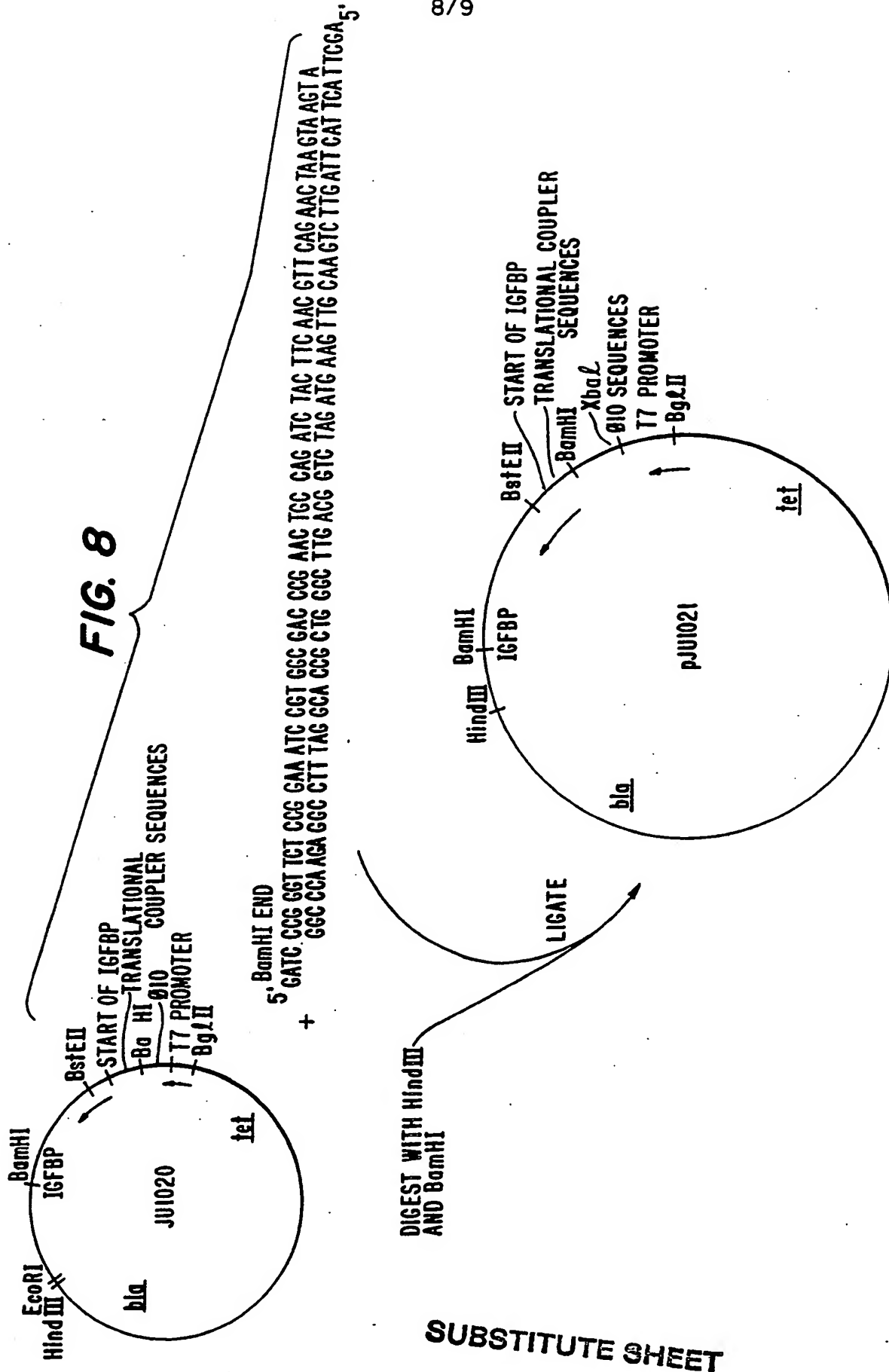
POLYLINKER SEQUENCE

5' GGATCCGAATTCATCGATCCCGGGAAGCTTGGTACCGACTAGTAA 3'
   GCTTAAGTAGCTAGGGCCCTTCGAACCATGGCTGATCATTCTAG

BamHI EcoRI ClaI SmaI HindIII KpnI SpeI XhoII

(EcoRI/EcoRV JUNCTION)
T∅ XhoII
POLYLINKER SEQUENCE
BamHI
∅10N-TERM T7P
BglII,SacII,SacI

bla
pJUI003
tet
COMPLETE
SalI

+ λgtII CLONE CONTAINING
THE IGFBP CODING SEQUENCE
DIGESTED WITH EcoRI AND
BstEII. ISOLATE THE
IGFBP CODING DNA FROM
CODER 26 THROUGH THE 3'
UNTRANSLATED REGION.

DIGEST pJUI003 WITH
BamHI AND EcoRI

+ THE SYNTHETIC OLIGONUCLEOTIDE
CODING THE FIRST 25 AMINO ACIDS OF
IGFBP, AN N-TERMINAL METHIONINE
AND A TRANSLATIONAL COUPLER.

IGFBP START

LIGATE   5' BamHI END   30   60
         GAT CCG ATC GTG GAG GAT GAT TAA ATG GCT CCG TGG CAG TGC GCA CCG TGC TCC GCA GAA
         GC TAG CAC CTC CTA CTA ATT TAC CGA GGC ACC GTC ACG CGT GGC ACG AGG CGT CTT

                                        90
         AAA CTG GCT CTG TGC CCG CCG GTT TCC GCA TCC TGC AGC GAG 3'
         TTT GAC CGA GAC ACG GGC GGC CAA AGG CGT AGG ACG TCG CTC CAGTG 5'
                                                            BstEII END

EcoRI   BamHI
HindIII   IGFBP

bla   BstEII
                                 START OF IGFBP
pJUI020   BamHI   TRANSLATIONAL COUPLER
                                 ∅10   SEQUENCES
                                 T7 PROMOTER
                                 BglII
tet

FIG. 7

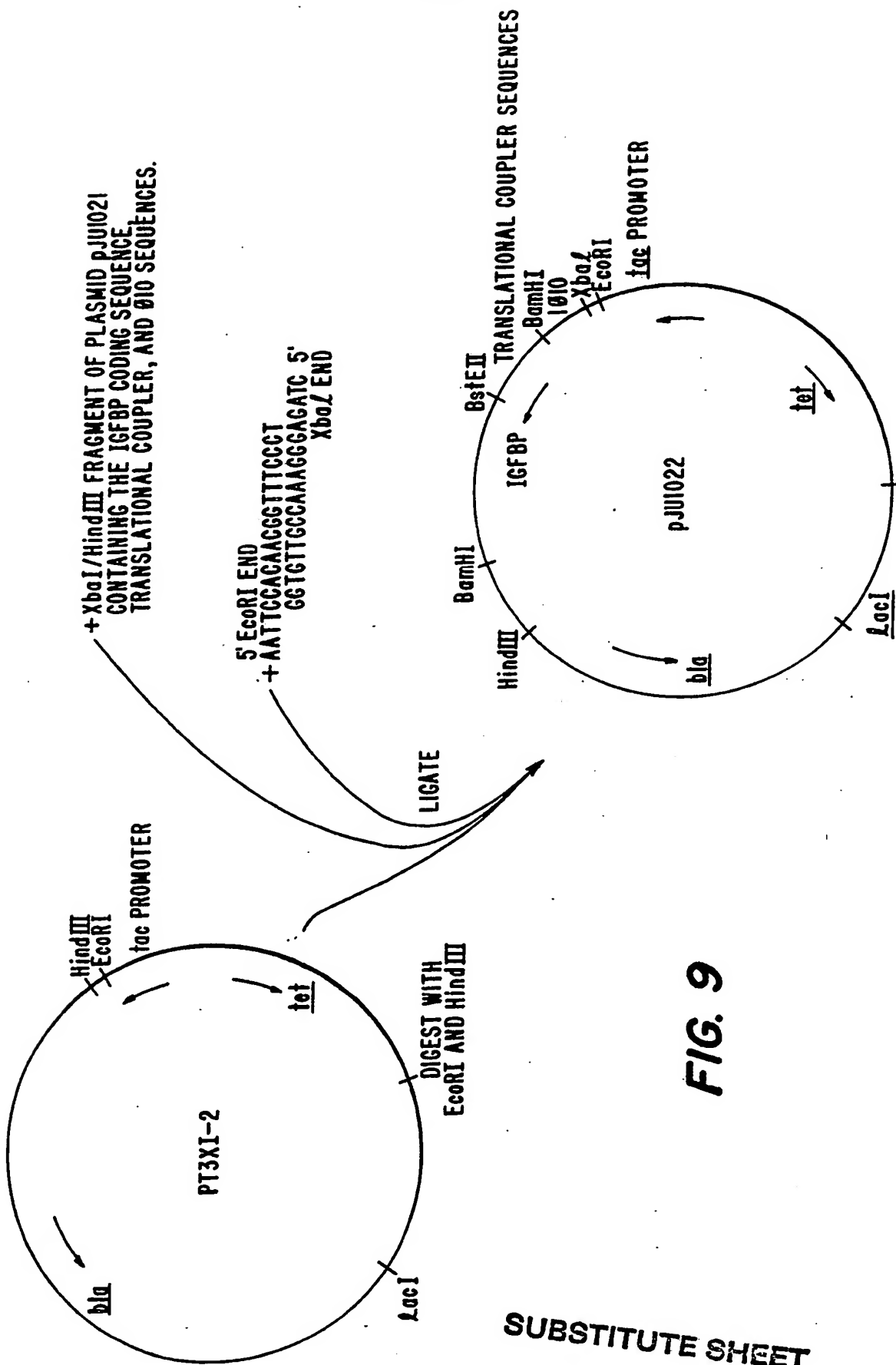**SUBSTITUTE SHEET**

FIG. 8

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FIG. 9

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01885

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07K 15/14; C07K 13/00; C12N 15/00
US CL.: 435/68; 530/380; 435/172.3; 530/395; 536/26

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	435/68; 435/172.3; 536/26 530/303; 530/350; 530/380; 530/395; 935/11; 935/24

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

BIOSIS, APS Computer Searches.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X Y	The Journal of Clinical Endocrinology & Metabolism, Vol. 66, No. 1, issued January 1988 (Baltimore, MD, USA), Rutanen et al, "Soluble 34K Binding Protein Inhibits the Binding of Insulin-like Growth Factor I to Its Cell Receptors in Human Secretory Phase Endometrium: Evidence for Autocrine/Paracrine Regulation of Growth Factor Action", pages 173 to 180.	1-3,15 1-15
X Y	The Proceedings of the National Academy of Sciences of the USA. Vol. 84. issued May 1987 (Washington, DC., USA), Elgin et al, "An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-1", pages 3254 to 3258.	1-3,15 1-15

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 August 1988	2 3 SEP 1988

International Searching Authority	Signature of Authorized Officer
ISA/US	JEFF P. KUSHAN

Category *	Citation of Document, with Indication, where appropriate, of the relevant passages	Relevant to Claim No
X̲ Y	Biochemical and Biophysical Research Communications, Vol. 148, No. 2, issued 29 October 1987 (Duluth, MN, USA), Bar et al, "Production of IGF-Binding Proteins by Vascular Endothelial Cells", pages 734 to 749.	1-3,15 ‾‾‾‾‾ 1-15
X̲ Y	Biochemical and Biophysical Research Communications, Vol. 139, No. 3, issued 30 September 1986 (Duluth, MN, USA), Baxter et al, "Growth hormone-dependent insulin-like growth factor (IGF) binding protein from human plasma differs from other human IGF binding proteins," pages 1256 to 1261.	1-3,15 ‾‾‾‾‾ 1-15
X̲ Y	Biochemical and Biophysical Research Communications, Vol. 147, No. 1, issued 31 August 1987 (Duluth, MN, USA), Baxter et al, "Binding proteins for insulin-like growth factors in adult rat serum. Comparison with other human and rat binding proteins", pages 408 to 415.	1-3,15 ‾‾‾‾‾ 1-15
X̲ Y	The Journal of Biological Chemistry Vol. 261, No. 24, issued 25 August 1986 (Baltimore, MD, USA), Mottola et al, "Purification and Amino-terminal Sequence of an Insulin-like Growth Factor-Binding Protein Secreted by Rat Liver BRL-3A Cells", pages 11180 to 11188.	1-3,15 ‾‾‾‾‾ 1-15
Y	US, B, 4,716,112 (PANAYOTATOS), issued 29 December 1987; see entire document, especially abstract and figure.	4-15
Y	US, B, 4,394,443 (WEISSMAN ET AL), issued 19 July 1983, see entire document.	4-15

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)